NEURAL RESPONSES TO INJURY: PREVENTION, PROTECTION, AND REPAIR
Annual Technical Report
1994

Submitted by

Nicolas G. Bazan, M.D., Ph.D.
Program Director

Period Covered: 20 September, 1993, through 19 September, 1994

Cooperative Agreement DAMD17-93-V-3013

between

United States Army Research and Development Command (Walter Reed Army Institute of Research)

and

Louisiana State University Medical Center Neuroscience Center of Excellence

Neurochemical Protection of the Brain, Neural Plasticity and Repair

Project Director:
Nicolas G. Bazan, M.D., Ph.D.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	19 October, 1994	Annual Report: 9/20/93 - 9/19/94

4. TITLE AND SUBTITLE
Neural Responses to Injury: Prevention, Protection, and Repair (Cooperative Agreement # DAMD17-93-V-3013)

5. FUNDING NUMBERS
97304000003758119
61110200000415000
AXZAC1KUF00000000
0AXZA00S18064
-AND-
21220400000275811
9611102H41ZZ41500
0ZYIZC1KUF0000000
00ZYIZ00S18064

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U. S. Army Research Office
P. O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSORING/ MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The LSU Neuroscience Center is a comprehensive, multidisciplinary, and transdepartmental entity that unites fundamental neurobiology and the clinical neurosciences in the common goal of elucidating the workings of the brain and contributing to the treatment of currently incurable diseases of the nervous system. The objective of the present program is to find solutions to neuroscience-related problems of interest to the U.S. Army Medical Research and Development Command. The program is focused on exploiting novel neuroprotective strategies that lead to prevention of and repair after neural injury. Converging approaches using state-of-the-art tools of cell biology, neurochemistry, neuroimmunology, neurophysiology, neuropharmacology, molecular biology and virology are proposed. Over the next four years, this program aims to: 1) carry out seven research projects in the basic and clinical neurosciences; 2) expand central, shared facilities with the addition of highly specialized instrumentation not currently available to our scientists; 3) develop laboratory space to permit the physical consolidation and coordination of this research effort; and 4) institute a coordination unit to monitor, facilitate, and administrate the cooperative research programs, as well as to meet the associated budgetary, human resources, facilities, and communications needs for the attainment of the proposed program goals.

14. SUBJECT TERMS

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

This Technical Report covers the progress made in the first year of this Cooperative Agreement in one project of the original proposal. We hope that this format of the report will facilitate its handling. The table of contents of all the projects has been included in each volume as well letters by members of External Advisory Committee of the LSU Neuroscience Center who have conducted an initial review of the work done supported by this Cooperative Agreement.

Nicolas G. Bazan, M.D., Ph.D.
Director, LSU Neuroscience Center
Program Director, USAMRDC Cooperative Agreement

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4. The frequency of exposure to morphine differentially affects CTL activity in alloimmunized mice.

Manuscripts:

1. Carr DJJ, Carpenter GW, Garza HH, Baker ML, Gebhart BM (in press) Cellular mechanisms involved in morphine-mediated suppression of CTL activity. In: *The Brain Immune Axis in Substance Abuse* (Sharp, Friedman, Maddin and Eisenstein, eds), Plenum Press.
2. Carpenter GW and Car DJJ (submitted) Pretreatment with β-funaltrexamine blocks morphine-mediated suppression of CTL activity in alloimmunized mice.
3. Carr DJJ and Carpenter GW (submitted) Morphine-induced suppression of spenic CTL activity in alloimmunized mice is not mediated through a δ-opioid receptor.
4. Carpenter GW, Garza HH, Gebhardt BM, Car DJJ (in press) Chronic morphine treatment suppresses CTL-mediated cytolysis, granulation and cAMP responses to alloantigen

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Project Director: Joseph Moerschbaecher, Ph.D.

Participating Scientists: Charles France, Ph.D.
Dennis J. Paul, Ph.D.
Jayaraman Rao, M.D.

Abstract:

1. International Symposium on Nicotine: The Effects of Nicotine on Biological Systems II: Bienvenu B, Kiba H, Rao J, and Jayaraman A. Nicotine induceds fos intensely in the parvocellular paraventricular nucleus and the lateral hypothalamus in rats.

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"Vision, Laser Eye Injury, and Infectious Diseases" . . . . .

Project Director: Herbert E. Kaufman, M.D.
Roger Beuerman, Ph.D.

Participating Scientists: Claude A. Burgoyne, M.D.
Emily Varnell
Mandi Conway, M.D.

Manuscripts

2. Chew SJ, Beuerman RW, Kaufman HE (in press) Real-time confocal microscopy of keratocyte activity in wound-healing after cryoablation in rabbit corneas. *Scanning* 16.

"Role of Growth Factors and Cell Signaling in the Response of Brain and Retina to Injury" . . . . .

Project Directors: Prescott Deininger, Ph.D.
Nicolas G. Bazan, M.D., Ph.D.

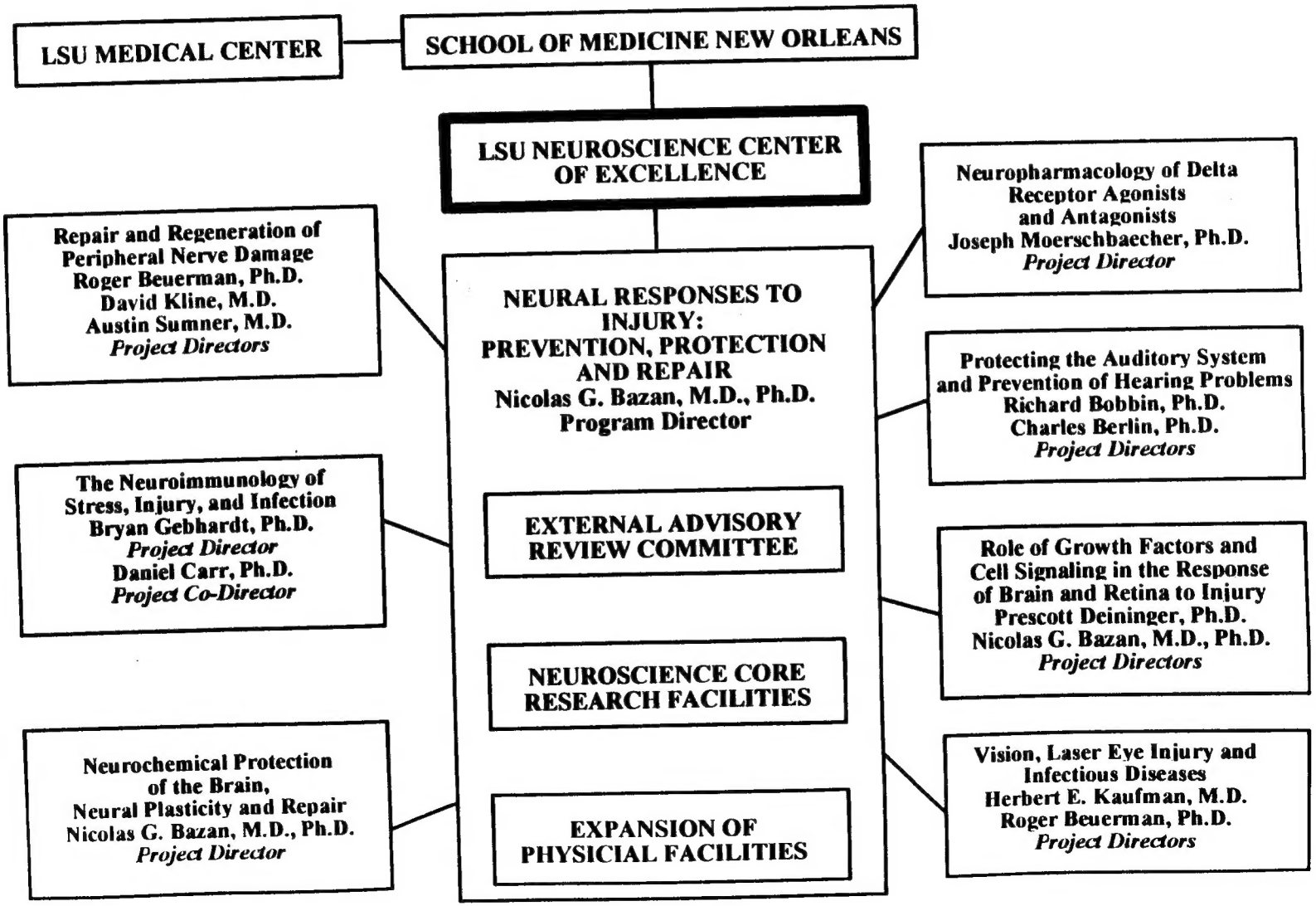
Participating Scientists: Julia Cook, Ph.D.
Haydee E. P. Bazan, Ph.D.
William C. Gordon, Ph.D.
Elena Rodriguez De Turco, Ph.D.
Victor Marcheselli, M.S.

Project Directors: Richard Bobbin, Ph.D.
Charles Berlin, Ph.D.

Participating Scientists: Sharon Kujawa, Ph.D.
Carlos Erostegui, M.D.
Douglas Webster,Ph.D.

Cooperative Agreement Between the US Army Medical Research and Development Command and The LSU Neuroscience Center of Excellence

DAMD17-93-V-3013 20 September, 1993 - 19 October, 1997 $13,860,000



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Neuroscience Center
Office of the Director

19 October, 1994

Commander
U.S. Army Medical Research and Development Command (USAMRDC)
ATTN: SGRD-RMI-S
Fort Detrick
Frederick, MD 21702-5012

Re: Annual report, Cooperative Agreement No. DAMD17-93-V-3013
Neural Responses to Injury: Prevention, Protection, and Repair

Dear Sir,

Please find enclosed the original and five copies of the first annual report for the Cooperative Agreement, referenced above, between the USAMRDC and the Louisiana State University Medical Center School of Medicine, Neuroscience Center of Excellence. This report represents the research carried out during the first year of this agreement (20 September, 1993, to date). It is organized per project, each corresponding to a chapter of the original application.

In addition to the research conducted in the first year of this agreement, the planning for the two additional floors of research space which are to be added to the Lions/LSU Clinics Building, 2020 Gravier Street, New Orleans, LA, has been completed, including all specifications necessary for the start of bidding. Enclosed is one copy each of the program manual (1 vol.) and the project manual (3 vols.) which has been generated by Cimini, Meric and Duplantier, Architects and Planners, for bidding purposes. It should be noted that there will actually be three floors constructed in this one project, two as funded by this Cooperative Agreement and one which is funded by LSU to be used by the School of Medicine for other purposes.

As planned, I arranged to have three meetings between the LSU investigators and their counterparts in the Army to provide program briefings for the work that they were planning to conduct under this agreement as well as to exchange ideas and information of mutual interest. The agendas for each of these meetings are enclosed. These provided both the LSU scientists and those of the Army the opportunity to discuss the work being done, the direction, and the significance to problems of interest to the Department of Defense.

On 2 December, 1993, several of our investigators, excluding the Auditory and Laser/Vision groups, met at the Walter Reed Army Institute of Research, Washington, D.C., with Drs. Frank Tortella, Joseph Long, Mark DeCoster and Jit Dave. These discussions revolved around the neurochemical and neuropharmacological aspects of the program project and provided a forum for the Army scientists tobegin interactions and exchange of information with our investigators.

On 31 January, 1994, the LSU auditory physiology group, represented by Drs. Charles Berlin and Richard Bobbin, and I met at Fort Rucker, AL, with Dr. Kent Kimball and Dr. Ben T. Mozo. These meetings involved presentations and discussions about the protection of the auditory system and prevention of hearing problems in humans.

The LSU investigators involved with the vision research, composed of Dr. Herbert Kaufman, Dr. Roger Beuerman and myself, met on 7 February, 1994, at Brooks Air Force Base, San Antonio, TX. These scientists and those of the Ocular Hazards Research Unit of the US Army Medical Research Detachment made presentations and conducted discussions focused on protection from, repair of, and prevention of laser injuries, specifically to the eye. Each of these information exchanges provided very useful direction and advice for the LSU investigators. These workshops will be conducted annually for the term of this agreement.

At the end of the first year of this program, as planned, I requested that two of the members of the External Advisory Committee of the LSU Neuroscience Center, Dr. Dennis W. Choi, Jones Professor and Head of the Department of Neurology, Washington University School of Medicine, and Dr. Fred Plum, Anne Parrish Titzell Professor and Chairman of the Department of Neurology, Cornell University Medical College, provide a critical review and a written report of the progress of the research accomplished under this Cooperative Agreement. Dr. Choi was given a copy of this annual report and subsequently made a site visit on 15 September, 1994, to the LSU Neuroscience Center. (The agenda for his meeting is attached.) At that time he met with a number of the investigators and administrators involved with whom he discussed many facets of the research being performed under this Agreement. His opinion of the work being done is attached.

Dr. Fred Plum made a site visit on 26 September, 1994, having also been provided previously with a copy of this annual report. He was also given the opportunity to examine the research and other progress made under this agreement and his written critique is also attached. Please note that, near the end of his letter (bottom of page two, first four paragraphs of page 5), Dr. Plum also included a description of projects not directly supported by the Cooperative Agreement but which are very positively impacted by any support of Neuroscience projects. The

reviewers were very complimentary of the positive consequences resulting from this support.

We are very pleased with the progress that has been made. We would like to thank you for the assistance you have given us. Please let me know if there is any further information that I can provide you.

Sincerely,

Nicolas G. Bazan, M.D., Ph.D.
Villere Professor of Ophthalmology,
Biochemistry and Molecular Biology,
and Neurology
Director, LSU Neuroscience Center

NGB/eht
enclosures

JOINT WORKSHOP ON "NEURAL RESPONSES TO INJURY: PREVENTION, PROTECTION AND REPAIR"

Sponsored by the LSU Neuroscience Center and Walter Reed Army Institute of Research, Department of Medical Neurosciences

December 2, 1993
Building 40, Room 2133

"Overview of LSU Program" **N. Bazan**	9:00
"Repair and Regeneration of Peripheral Nerve Damage" **R. Beuerman, D. Kline, J. England**	9:20
"The Neuroimmunology of Stress, Injury and Infection" **D. Carr**	10:10
Break	10:20
"Neurochemical Protection of the Brain, Neural Plasticity and Repair" **N. Bazan**	10:40
"Neuropharmacology of Delta Receptor Agonists and Antagonists" **J. Moerschbaecher**	11:15
"Stress and the Dopamine System" **J. Rao**	11:45
Box Lunch Served ($2.00 each)	12:00
"Role of Growth Factors and Cell Signaling in the Response of Brain and Retina to Injury **N. Bazan and J. Cook**	12:10
"An Overview of Neuropharmacology Research at WRAIR on Nervous System Injury and Protection" **Frank Tortella**	13:00
"Animal Models of Spinal Cord Injury and Mechanisms of Blood Flow Changes" **Joseph Long**	13:30
"Evaluation of Excitatory Amino Acids in Neuronhal Cell Culture" **CPT DeCoster**	13:50
"Molecular Biology of Nervous System" **Jit Dave**	14:10
Overall Discussion	14:30
Adjourn	15:00

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Joint Workshop on Neural Responses to Injury:
Prevention, Protection and Repair
Walter Reed Army Institute of Research, Dept. of Medical Neuroscience
U.S. Army Aeromedical Research Laboratory, Fort Rucker, AL
SCHEDULE FOR JANUARY 31, 1994

January 30
12:00 PM - depart New Orleans by car
Hotel: **Comfort Inn, 615 Boll Weevil Circle, Enterprise, AL 36330**
Tel. 205-393-2304, Fax. 205-347-5954

January 31
Visiting - Dr. Kent Kimball, Director, Plans and Programs, USAARL
Dr. Ben T. Mozo, Research Physicist, USAARL
Fort Rucker, AL 36362-5292
Tel. (205) 255-6917, Fax. (205) 255-6937

9:00 AM - Welcome
9:20 AM - Overview of LSU Program - **Nicolas G. Bazan**
9:45 AM - Protection the Auditory System and Prevention of Hearing Problem via Efferent Activation in Humans - **Charles Berlin**
10:30 AM - Break
11:00 AM - Prevention of Hearing Problems in Animals - **Richard Bobbin**
12:00 PM - General Discussion and Lunch
13:00 PM - Adjourn

OCULAR HAZARDS RESEARCH
U.S. ARMY MEDICAL RESEARCH DETACHMENT
7914 A DRIVE (Bldg 176)
BROOKS AIR FORCE BASE, TEXAS 78235-5138

February 7, 1994

Leave New Orleans on Continental flight #1445 at 6:00 PM, arrive San Antonio on Continental flight #1120 at 8:53 PM.

Hyatt Regency San Antonio
123 Losoya St., San Antonio, TX 78205
Confirmation #HY0000605552

February 8, 1994

8:30 *Overview of USAMRD program*
Bruce Stuck, Director, USAMRD

8:45 *Review of Accidental Laser Exposures and Human Tissue Response*
Donald Gagliano, Commander, USAMRD

9:00 *Overview of LSU Program*
Nicolas G. Bazan, Director, LSU Neuroscience Center

9:10 *The Program: Vision, Laser Eye Injury, and Infectious Diseases*
Herbert Kaufman, Chairman, Ophthalmology Dept. LSU

10:00 *Confocal Approach to Cellular Reactions in Wound Healing and of the Lamina Cibrosa.*
Roger Beuerman of the LSU Neuroscience Center

10:30 **BREAK AND LAB TOUR**

10:50 *Neurochemical Protection of the Brain, Neural Plasticity, and Repair*
Nicolas Bazan, Director, LSU Neuroscience Center

11:40 *Basic Fibroblast Growth Factor (bFGF) Treatment of Laser-Injured Retina*
Steven T. Schuschereba, Chief, Biology Section, USAMRD

12:10 *Role of Growth Factors and Cell Signaling in the Response of Brain and Retina to Injury: Focus on the Retina*
Nicolas Bazan, Director, LSU Neuroscience Center

12:50 **LUNCH**

2:50 Depart San Antonio on Southwest flight #803

5:55 Arrive New Orleans on Southwest flight #1055

LETTERS FROM MEMBERS OF THE EXTERNAL ADVISORY COMMITTEE

WASHINGTON UNIVERSITY SCHOOL OF MEDICINE
AT WASHINGTON UNIVERSITY MEDICAL CENTER

NEUROLOGY

Dennis W. Choi, M.D., Ph.D.

Andrew B. and Gretchen P. Jones Professor and Head
Neurologist-in-Chief, Barnes Hospital

October 17, 1994

Nicholas G. Bazan, MD, PhD
Director, LSU Neuroscience Center
School of Medicine in New Orleans
Louisiana State University Medical Center
2020 Gravier Street, Suite "B"
New Orleans, LA 70112-2234

Dear Nick:

Thank you for the invitation to visit LSU on September 15 and review early progress made under the LSU Neuroscience Center of Excellence Cooperative Agreement with the U.S. Army Medical Research and Development Command.

You have assembled an impressive array of faculty researchers to study diverse aspects of nervous system injury. Overall, I find the individual projects to be thoughtful and well chosen. With you as director, I am sure that they will be most ably integrated. Your project 3 "Neurochemical Protection of the Brain, Neuroplasticity and Repair" is in my view the clear focal point of the overall program. The identification of new PAF antagonist drugs capable of regulating excitatory synaptic transmission and excitotoxic central nervous system injury, is an attractive and attainable goal. The novel pharmacology theme is also well developed in Dr. Moerschbaecher's Section 4 "Neuropharmacology of Delta Receptor Agonist and Antagonist". Involvement of clinician-investigators in clinical departments, such as Dr. Sumner in Project 1 or Dr. Kaufman in Project 5 are strengths of the program that will enhance its ability to identify human therapeutic interventions.

Progress in the first months of operation appears to be on target. Substantial synergy can be expected between the research programs specifically outlined in this collaborative agreement, and the larger intellectual framework formed the LSU Neuroscience Center of Excellence. Your role as director of both efforts is a vital feature that will ensure maximization of this synergy. In summary, I am most enthusiastic about this LSU-U.S. Army Cooperative Agreement, both for its specific merit and as a prototype mechanism for facilitating effective collaboration between academic and military institutions.

Best regards.

Sincerely,

Dennis Choi

Box 8111
660 South Euclid Avenue
St. Louis, Missouri 63110
(314) 362-7175 • FAX (314) 362-2826

525 EAST 68th STREET NEW YORK N.Y. 10021

THE NEW YORK HOSPITAL-CORNELL MEDICAL CENTER

FRED PLUM M.D., CHAIRMAN
ANNE PARRISH TITZELL PROFESSOR OF NEUROLOGY
CORNELL UNIVERSITY MEDICAL COLLEGE
NEUROLOGIST-IN-CHIEF
THE NEW YORK HOSPITAL- CORNELL MEDICAL CENTER
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September 28, 1994

Nicholas G. Bazan, M.D., Ph.D.
LSU Neuroscience Center
2020 Gravier Street
Suite B
New Orleans, LA 70112-2234

Dear Dr. Bazan:

I am pleased to submit this reviewer's report of a Cooperative Agreement between the LSU Neuroscience Center and the US Department of the Army entitled, "Neural Response to Injury: Prevention, Protection and Repair" (henceforth designated as "Injury Study"). The agreement will span four years of effort by the LSU Center; this report describes progress obtained during its first year, extending from September 1, 1993 to August 31, 1994.

Nicholas G. Bazan, M.D., Ph.D. both directs the LSU Neuroscience Center of Excellence and serves as the Program Director of the Injury Study. In addition to Dr. Bazan's personal investigative efforts, seven additional study groups are engaged in research directly related to the Injury Study, as indicated in the administrative diagram attached to this report.

Dr. Bazan's outstanding personal and scientific qualities are the two most important factors in assuring the future success of the LSU-U.S. Army Cooperative Agreement. His leadership and intellectual "taste", as well as his joy in and dedication to brain science penetrate every aspect of the LSU Neuroscience Institute. His enthusiasm has spread to infect his colleagues and many other departments of the Medical School with his high scientific standards and integrity. His knowledge suffuses every dimension of basic neuroscience. His diplomacy and gentle handling of his staff creates their huge loyalty. His energy is contagious. Furthermore, he has the wonderful quality of scientific generosity: always ready to help and encourage others, he is entirely responsible for the continuously improving quality of young persons who are coming to LSU to learn and do important neuroscience.

In addition to the above, Dr. Bazan's specific research is internationally recognized as being of the highest caliber. His personal research contributions to the Injury Study during the past year reflects these high qualities in several ways. They have been published in the most competitively prestigious biomedical research journals. They also add new understandings to both the normal and potentially abnormal effects of the platelet-activating factor (PAF). PAF already is known to be a potent mediator of inflammatory and immune responses. What Bazan and his team now have found is that in low concentrations, PAF transmission may enhance memory and repair mechanisms in brain. Alternately, if released in excessively large concentrations or in combination with certain other molecules, PAF appears capable of causing immune-related tissue damage such as occurs with intense inflammation and/or the induction of genetic prostaglandin synthesis, a step that also may injure brain tissue. This fundamental research emphasizes the complexity and often bidirectional responses that may occur when injury strikes the brain. The results are important and illustrate the difficulties which must be overcome in establishing prevention, protection and repair of brain injuries.

Drs. Bazan and Prescott Deininger have succeeded in developing a series of transgenic mice expressing a dominant mutant of platelet derived growth factor (PDGF). Remarkably enough, the animals thus far have shown no major behavioral alteration under

normal developmental conditions. Their reaction to ischemia, seizures and other circumstances has not yet been tested.

Let me turn now to some of the other, supporting projects: **Drs. R. Benerman, D. Kline and A. Sumner** have made good progress in their studies of neurotrophic factors and other mechanisms in human and experimental neuromas resulting from blunt and crush nerve injuries. Basic fibroblast growth factor (bFGF) was the most prominent factor found in human post-nerve injury neuromas with other specific factors either absent or reaching only very low levels of concentration. More precisely analytic experiments await the analyses of fresh neuronal material from the experimental preparations.

Drs. Herbert Kaufman and Roger Benerman have made brilliant advances using confocal microscopy to examine the cellular details of the human retina. To a degree never before possible they have safely demonstrated in awake human subjects the acute pathophysiology of laser injuries to cornea and their early transformation into fibroblasts. Detailed identification of anterior chamber cells has been possible and current efforts are underway to examine at great magnification the optic disc itself. Ocular fungus and herpes infections can be identified immediately and without introducing foreign substances against the cornea or into the eye. Application of the tool should have an important place in clinically applied military medicine.

During the past year, the investigators also have pursued their earlier discovery that ambient chilling of monkeys latently infected with H. Simplex induces an acute recurrence of cutaneous herpes. Furthermore, chronic ingestion of the beta blocker, propanelol, has been found to ameliorate or prevent the active recurrence. Clinical trials of this important discovery must be pursued as it has important practical aspects.

During the year, the necessary work to establish and equip the glaucoma research laboratory was undertaken. Next year's report can be expected to provide research results from that laboratory.

Dr. Joseph Moerschbaecher and his colleagues in pharmacology have initiated preliminary studies on the influence of delta opoid agonists-antagonists on learning and antinociception. Somewhat surprisingly, the agent damps the CO_2 response of breathing but has no antinociceptive effect. The same investigator is analyzing how anxiogenic drugs affect dopamine neurons in the ventral tegmental area of the rodent brain.

In another preliminary approach, **Drs. H.W. Thompson et al** have initiated experiments passing retroviral gene carriers into the eye with externally applied eye drops, thereby developing a new approach to deliver protection against certain ophthalmologic infections or enhancing the potential success of corneal transplant.

Drs. Richard Bobbin and Charles Berlin, thanks to the DOD grant, have added an excellent postdoctoral student as well as important new equipment to their laboratory. The laboratory's principal subject of interest is to find mechanisms for preventing the audiologic damage produced by intense sound. In guinea pigs, this has been achieved by stimulating calcium-dependent mechanisms in cochlear neurons. In another study, the laboratory has found in human studies that during the delivery of loud, binaural sounds, men and women suppress the noise in opposite sided ears from each other.

The above individual achievements provide only a part of the considerable effort, enthusiasm and success that the U.S. Army grant has brought to the LSU Neuroscience Center of Excellence (NCE). The following steps forward can also be emphasized:

1) Morale in the LSU-NCE rides at high pitch, encouraging scientific collaboration and the generation of new ideas.

2) Funds have been granted to subsidize the necessary equipment and technical personnel to establish a brain bank. Presently, approximately 50 specimens are available in storage with the Center holding good clinical records of the preterminal illness.

3) A program of "starter" grants designed to assist young investigators in conducting merit-deserving, self designed research projects has been initiated.

4) A highly popular state-wide Graduate School outreach summer program has been successfully concluded, attracting a strong interest in neuroscience among gifted college students.

5) An interdisciplinary graduate program in neuroscience was initiated and strongly encouraged by the faculty during 1993-94. As a result, nearly all of the graduate students (including the new entering class) are of very good quality. Indeed, other participating departments say that the Neuroscience graduate students are the best among the LSU biological sciences programs.

Summary. Under the generous auspices of a U.S. Army Cooperative Agreement, the LSU Neuroscience Center of Excellence is not only thriving but headed for far greater future productivity than at any time in the past. The admirable success of the program depends heavily on the foresight, intelligence, creativity and energy of two outstanding scientists, Herbert Kaufman and, especially, Nicholas G. Bazan. Their achievements and those of their colleagues totally warrant continuation of support. Indeed, every indication is that their extramural, non-Army support will continue to grow, making the program stronger and stronger as the years elapse.

One serious problem remains - that of sufficient space in which to do the studies that Dr. Bazan and his colleagues already have conceived so well. Prompt attention to and effective application of must be given to the DOD funds already awarded to construct new research space which will greatly increase the LSU Neuroscience team's opportunities for creative discovery.

I and my colleagues on the External Advisory Board of the LSU Neuroscience Center of Excellence strongly endorse the quality and number of achievements that have come from the U.S. Army-LSU-NCE collaboration. Thanks to strong leadership for the Center and a high degree of internally high morale and interdependence within the Center, it can be anticipated that the Cooperative Agreement will have a major impact on national neuroscience research as well as the specific medical needs of the U.S. Army.

Sincerely,

Fred Plum, M.D.

FP/moc

NEUROCHEMICAL PROTECTION OF THE BRAIN, NEURAL PLASTICITY AND REPAIR

PAF is a Presynaptic Mediator of Excitatory Neurotransmittor Release

Neuroanatomical Correlation of PAF Antagonist-Affected Gene Expression

Traumatic Brain Injury

Project Director:

Nicolas G. Bazan, M.D., Ph.D.

Participating Scientists:

Geoffrey Allen, Ph.D.
Gary D. Clark, M.D.
Victor Marcheselli, M.S.
Hurst, Ph.D.
Leo Happel, M.D.
Walter Lukiw, Ph.D.

NEUROCHEMICAL PROTECTION OF THE BRAIN, NEURAL PLASTICITY AND REPAIR

The Kindling Model of Epileptogenesis and Synaptic Plasticity

The Kindling Model of Epileptogenesis and Synaptic Plasticity

Introduction

Kindling refers to a phenomenon in which repeated application of an initially subconvulsive electrical stimulus produces limbic and clonic motor seizures of progressively increasing severity (1,2). Once established, the increased excitability is lifelong. This lifelong increase suggests that kindling leads to neuronal reorganization and, thus, might be an appropriate model for the study of neuronal plasticity (3,4). Therefore, it is of interest to study the gene expression and morphological changes which accompany the kindling process and is also of intense interest to try to manipulate the system in an attempt to reverse these changes. To accomplish this we have developed a computer driven system for fast hippocampal kindling of the rat which will allow us to accomplish these goals.

Materials and Methods

Our kindling apparatus is a powerful new tool for investigating changes that occur as a result of kindling because of it's ability to produce large numbers of kindled animals costing minimal technician time. This fast kindling paradigm is accomplished over a 21-day period using male Sprague Dawley rats weighing 250-275g. These rats are implanted unilaterally with a stainless steel electrode in the left hippocampus. The animals are left to recover for 7 days after surgery. Following the animals' recovery, their after discharge threshold (ADT) is determined. This threshold is the minimum stimulus required to produce an electrical discharge. The ADT consistently falls within a range of 30 μAmps to 70 μAmps. After verification of a proper ADT the animals are given 12, 400 μAmp stimulations every 30 min lasting 10 sec each over a six

hour period. The animals are trained in this manner a total of 4 times every other day. Following this training period the animals are allowed to rest for seven days until hippocampal tissue is collected and analyzed by Northern Blot Analysis. A diagram of the kindling apparatus appears in figure 1.

The sequence of events that are followed in the kindling model are as follows:

Surgury

1. Surgery performed on 250g-275g S.D. male rats.

2. 350 µl Ketamine and 35 µl Xylazine are used for anesthesia. Halothane supplement is used when needed. Allow animal 10 min. after injection of ket/xyl mixture to fall into a deep surgical plane of anaesthesia. If animal shows any sign of arousal during surgery immediately use halothane.

3. Two screws and dental cement are used for securing of electrode to head. One screw is placed lateral to the central fissure the other is placed in the temporal bone. The screws are arranged in a manner which places them on either side of the electrode.

4. The stereotaxic coordinates, relative to Bregma, are 0.36 posterior, 0.49 lateral, 0.50 ventral.

5. Electrodes are cut to approx. 1 cm at a 45 degree angle.

6. The hole drilled to accept the twisted portion of the electrode is drilled with an 0-80 size drill bit. It is important to clear all bone chips out of hole before inserting the electrode.

7. The hole drilled to accept the single wire of the electrode is drilled with a 2mm drill bit.

8. It is very important to dry scull completely before application of the dental cement. There should be no bleeding at time of application.

After discharge threshold determination

1. The ADT threshold must be determined before any kindling protocol is initiated. The ADT threshold of a properly implanted rat should be between 30μAmp to 70μAmp.

2. For determination: begin stimulating at 20μAmp and increment by 10μAmp until a response is achieved (typical response amplitude of 2mV to 3mV). It is wise to continue testing for an afterdischarge until three consecutive stimulations elicit an EEG response.

Kindling Parameters

1. 12 stimulations a day every 30min at 400μAmp.

2. 10 sec. stimulation duration , 1 sec. repolarization time.

3. 50 HZ frequency.

4. Kindle animals every other day for 8 days.

Results

After repeated electrical stimulation, animals progressively exhibit increasingly epileptiform discharges, as well as more intense behavioral responses. The typical progression of an after-discharge is shown in figures 2 and 3. Figure 2 displays an EEG following stimulation on the fourth day. This response is markedly more epileptiform than the response of the same animal on the first day of training, as shown in figure 3.

Finally, because of the inherent relationship shared between kindling, neuronal reorganization, and long-term potentiation, it will be interesting to study genes thought to play key roles in these processes. One gene product of interest is protein kinase C because of its' implication in long-term potentiation. Currently, we are working on setting up the proper

techniques needed to visualize changes in the expression of different isoforms of PKC. In addition, we have began chronic infusion of substances into the rat brain. Through the use of a mini-osmotic pump, PAF-antagonists are being infused into the lateral ventricles and behavioral and gene expression changes are being observed during and following kindling.

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Figure Legends

Figure 1. Diagram showing the current Kindling Apparatus and switching scheme.

Figure 2. After discharge exhibited by animal #11 following the third stimulation at 400 μAmps on the fourth day of training.

Figure 3. After discharge exhibited by animal #11 following the fourth stimulation at 400 μAmps on the first day of training.

figure 1A KINDLING APPARATUS

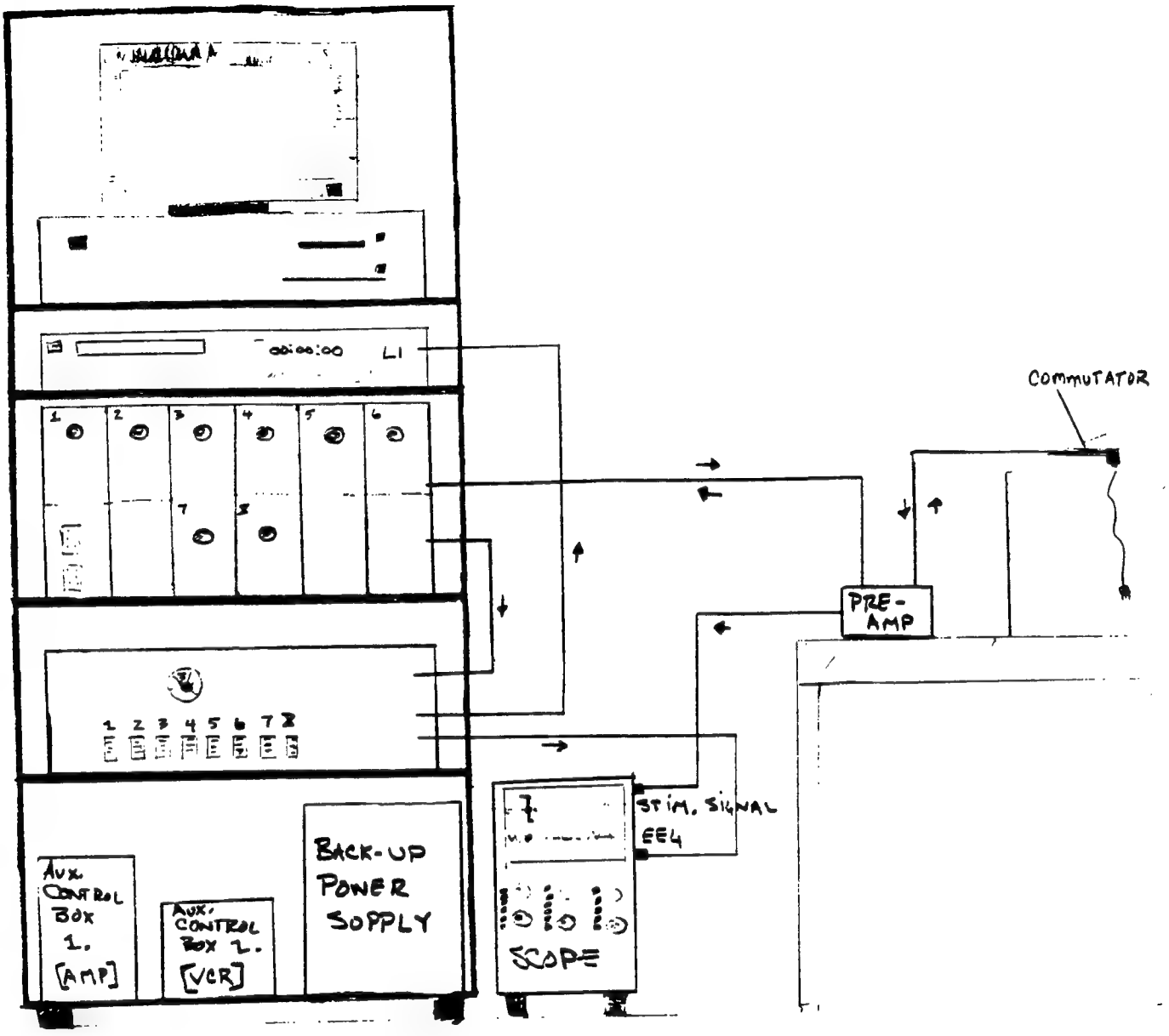
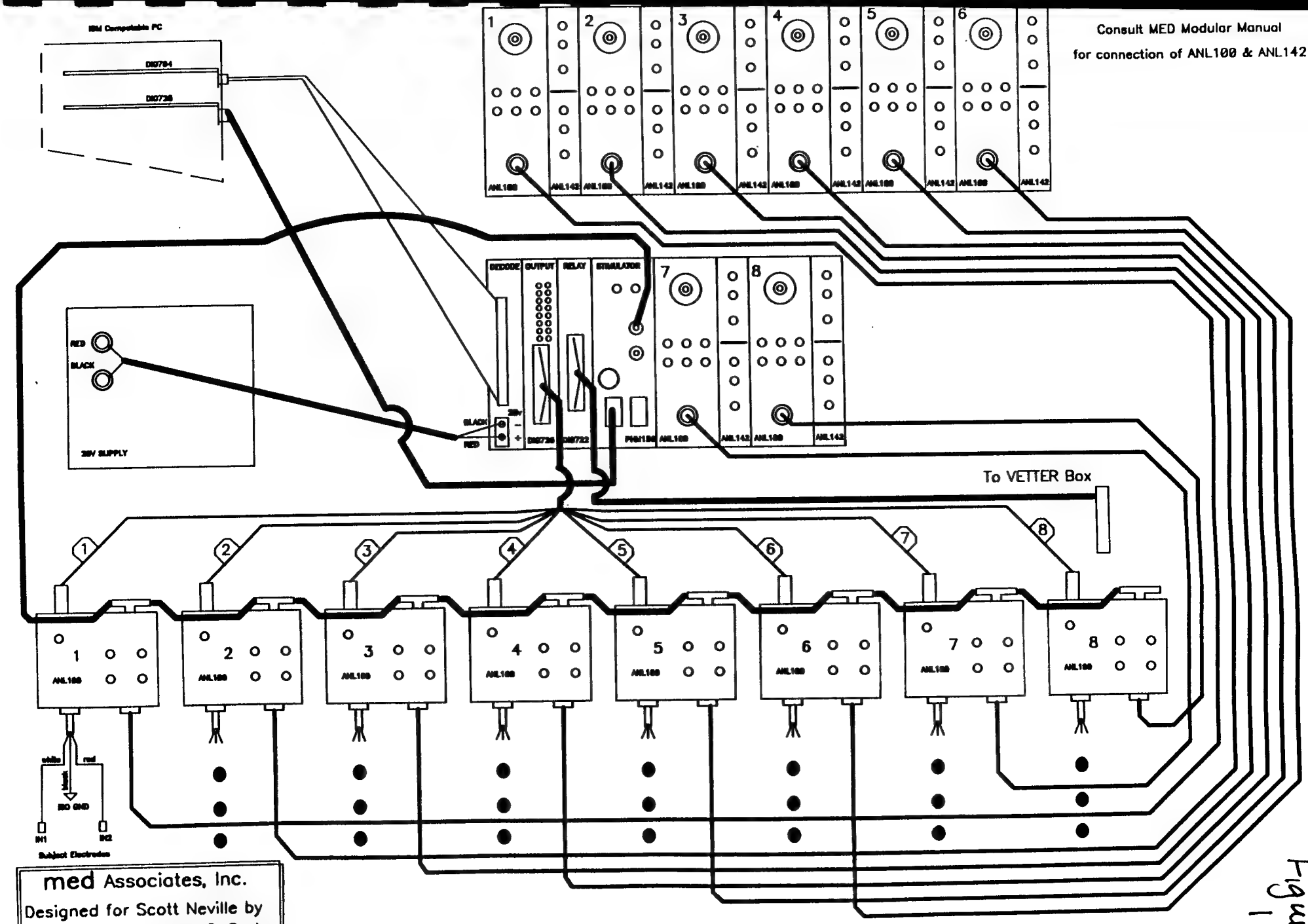


Figure 1a and 1b Diagram showing the current Kindling Apparatus and switching scheme.



WIRING DIAGRAM – LSU CUSTOM PACKAGE

Figure 29
1B

Figure 2, A through H. After discharge exhibited by animal #11 following the third stimulation at 400 μAmps on the fourth day of training.

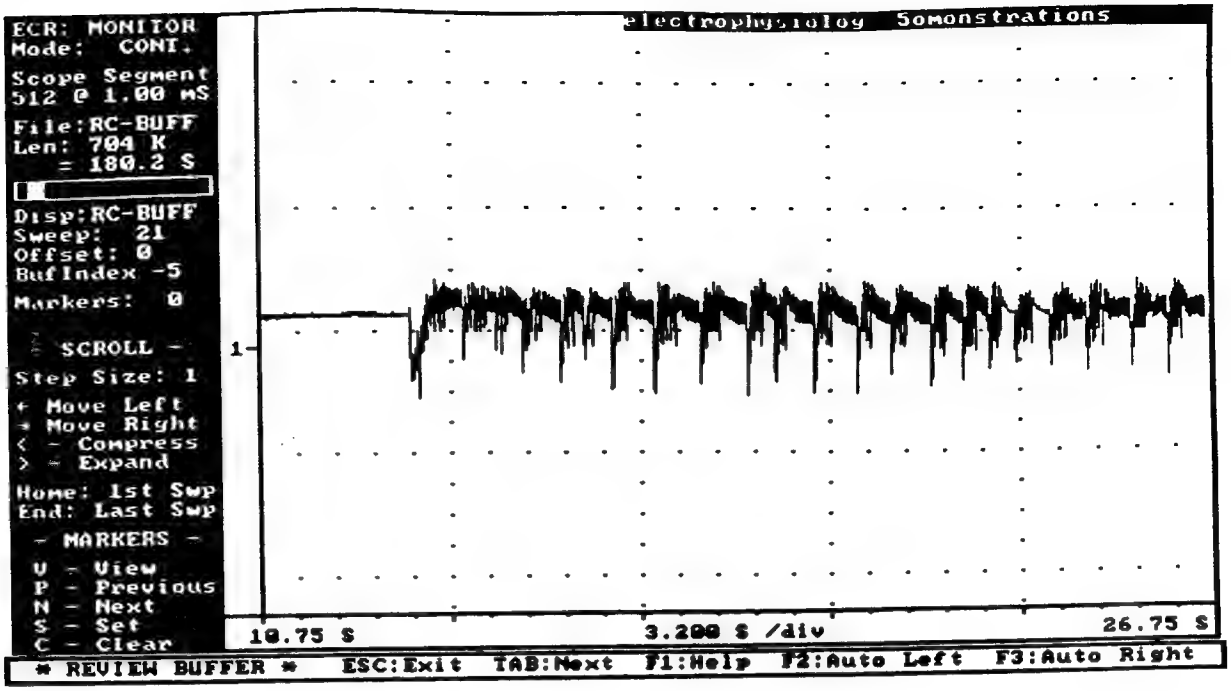


Figure 2B

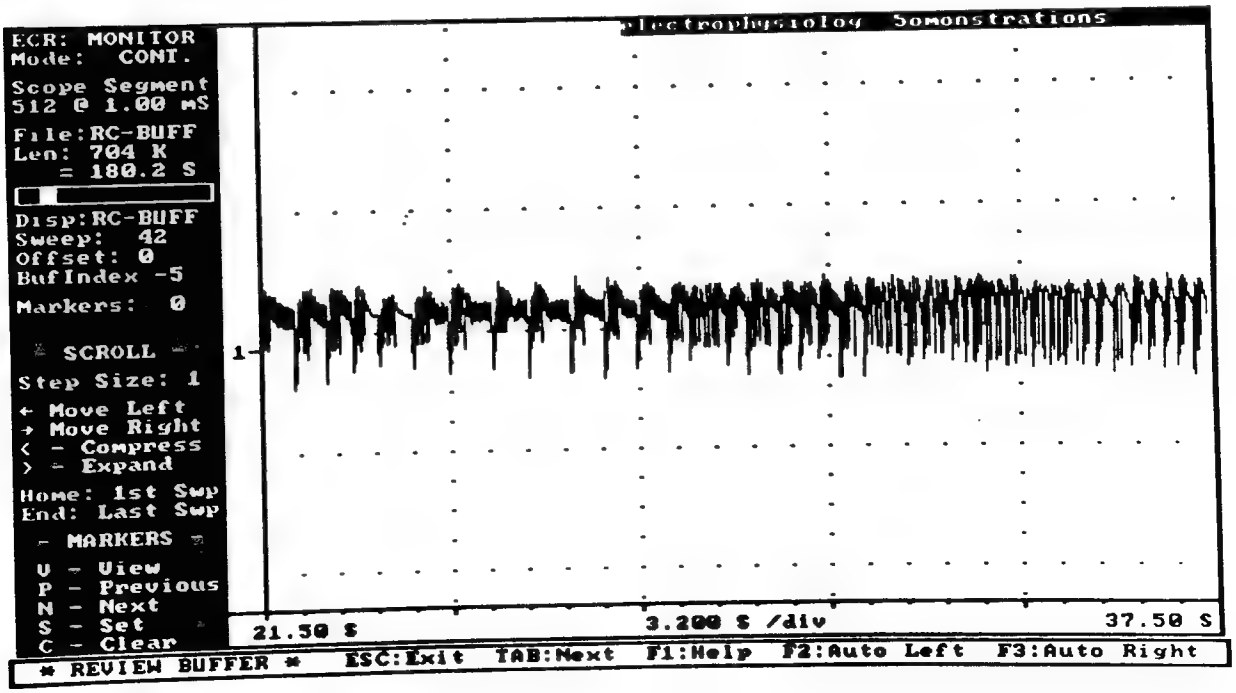


Figure 2C

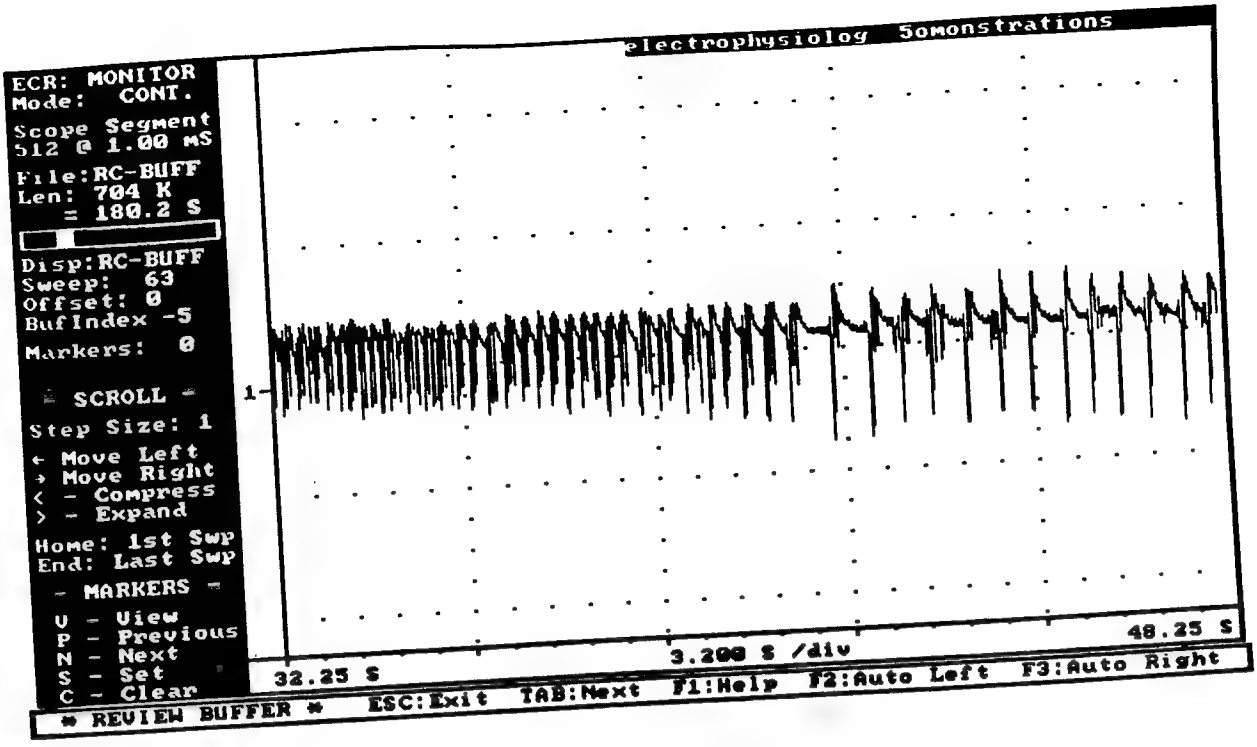
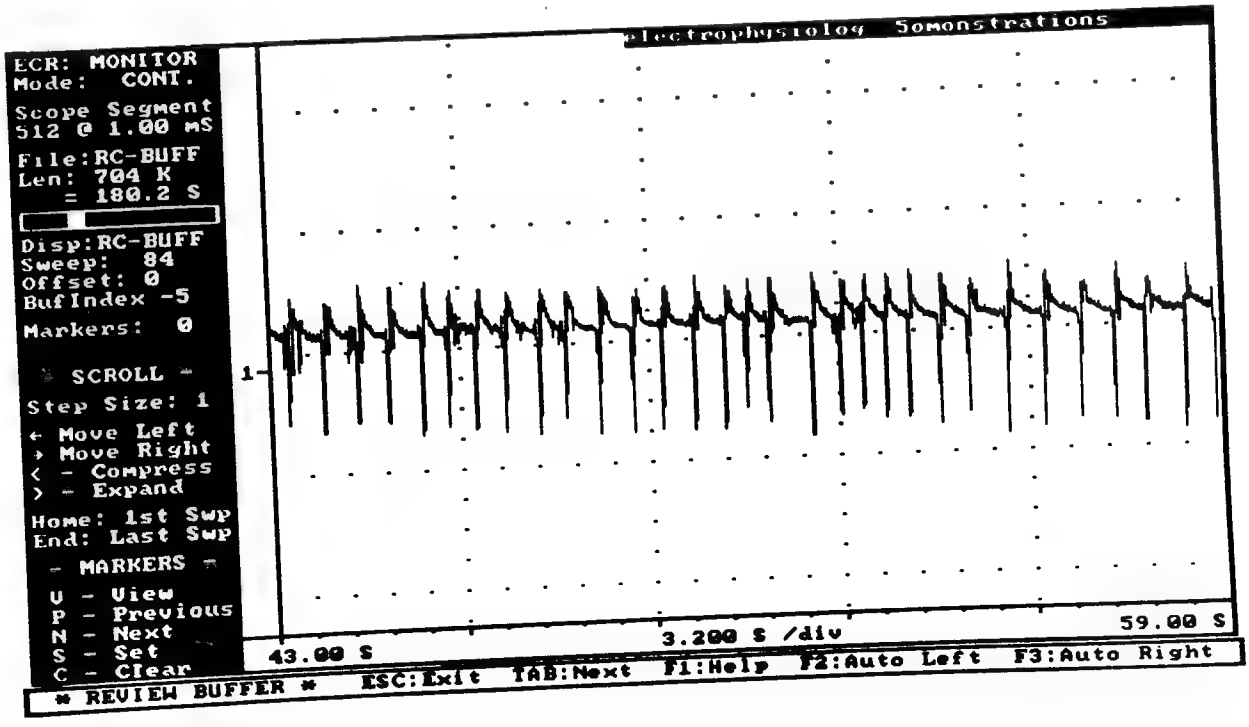


Figure 2D



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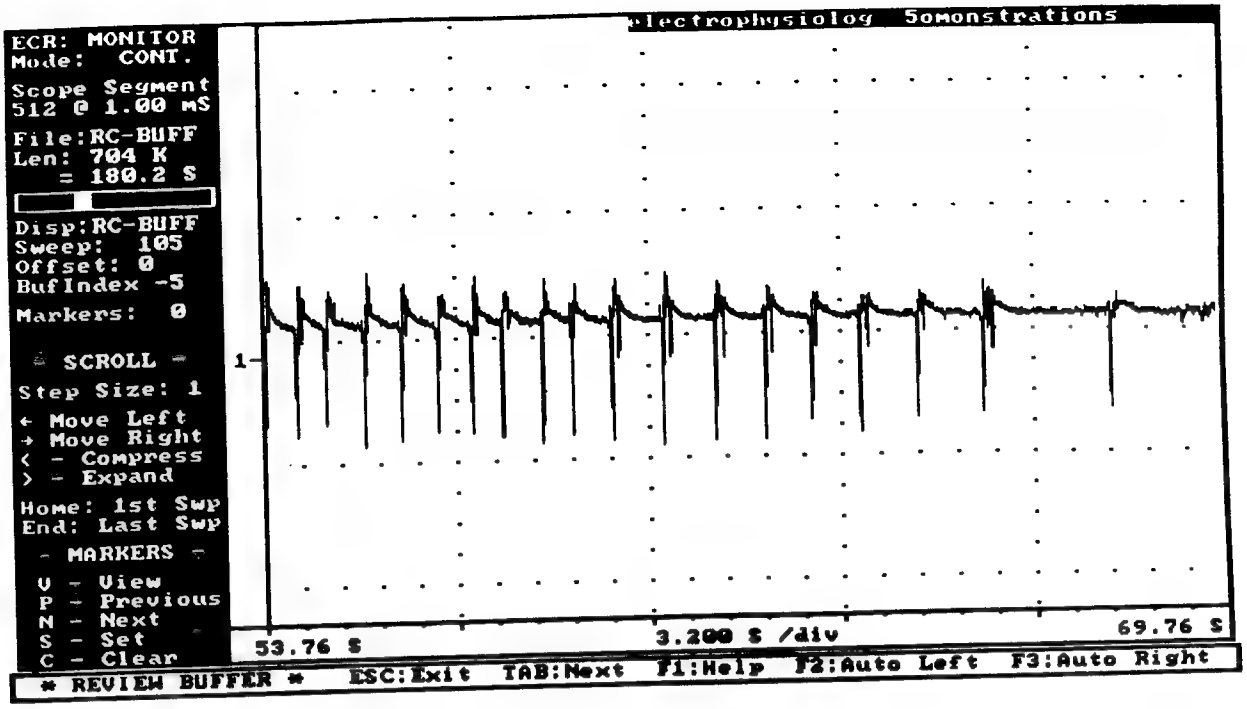


Figure 2F

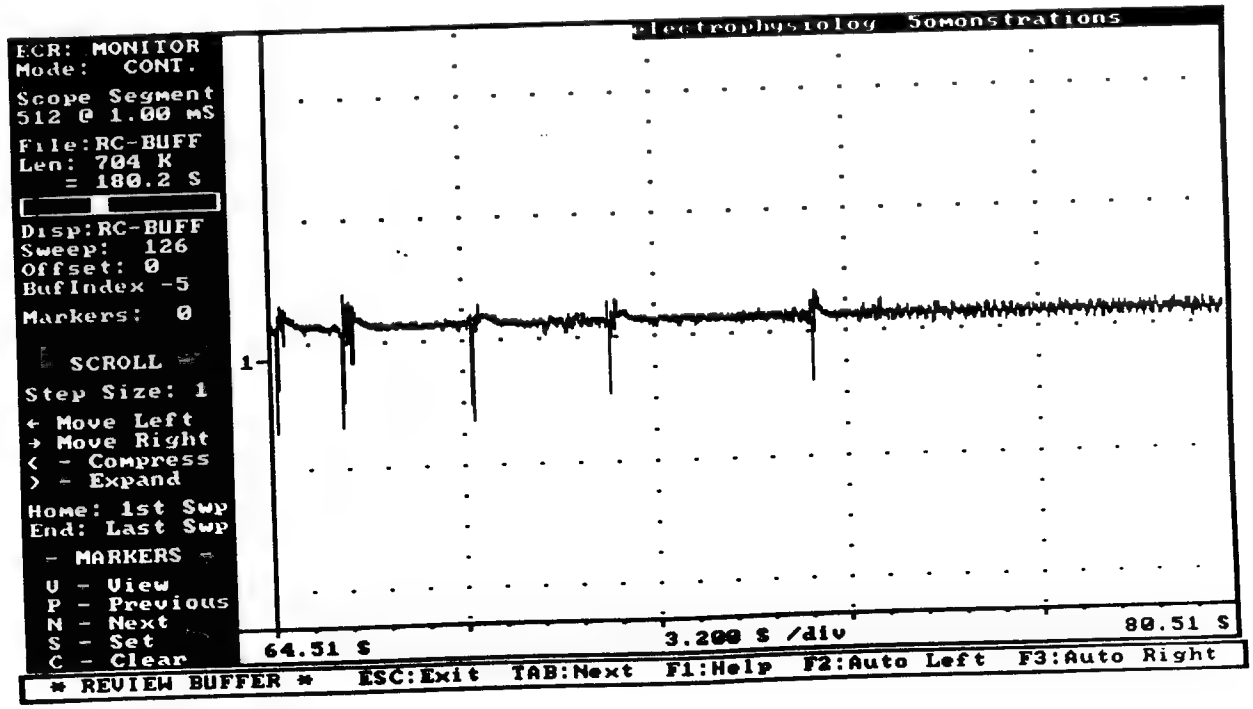


Figure 3, A through J. After discharge exhibited by animal #11 following the fourth stimulation at 400 μAmps on the first day of training.

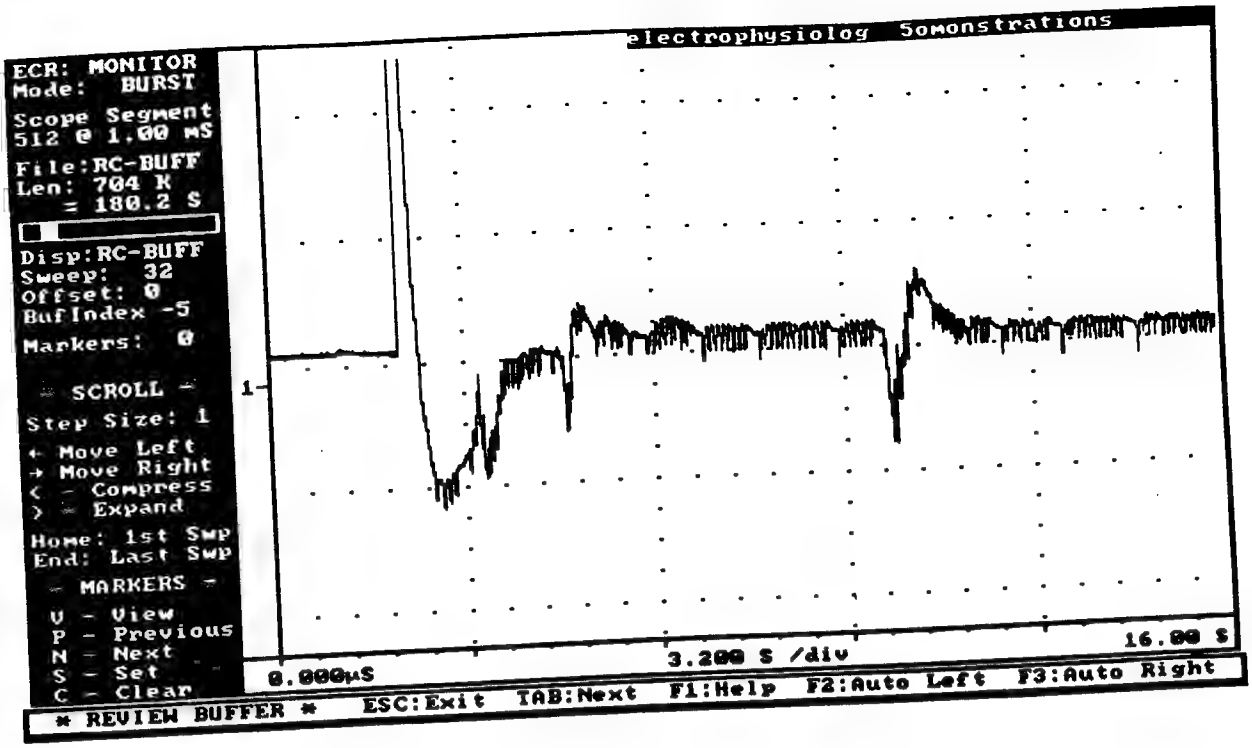
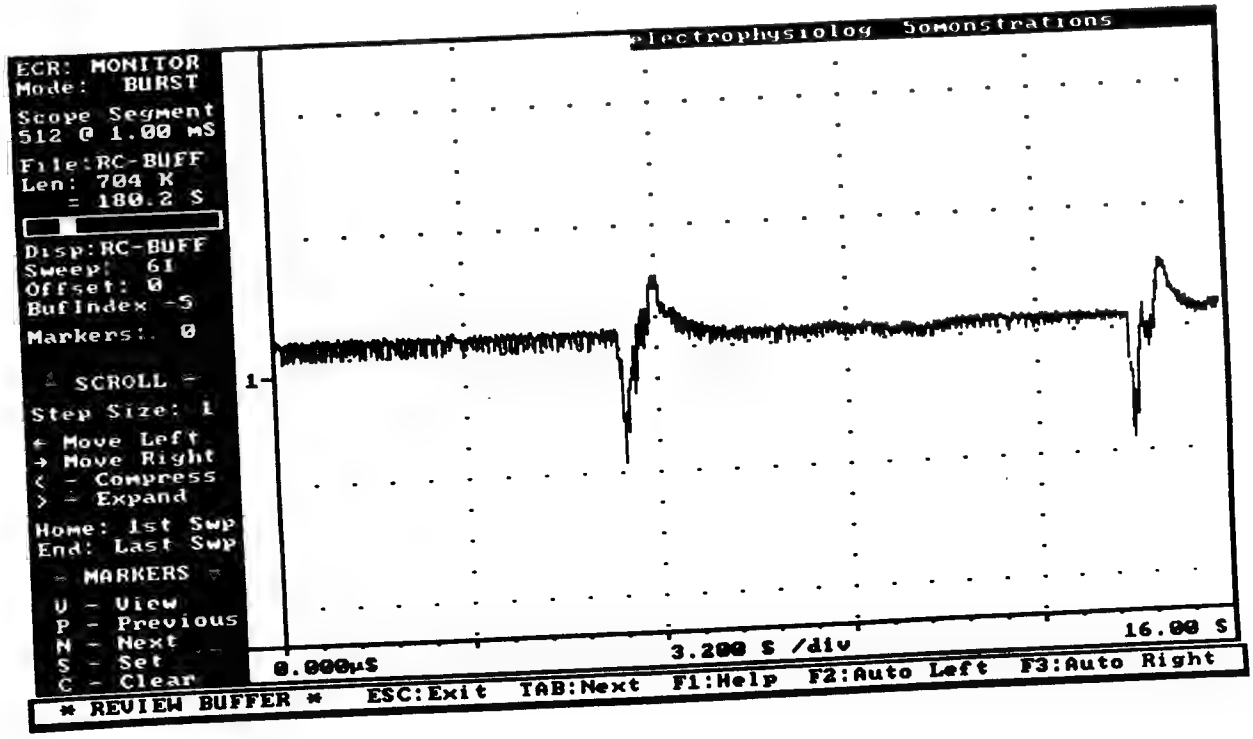


Figure 3B



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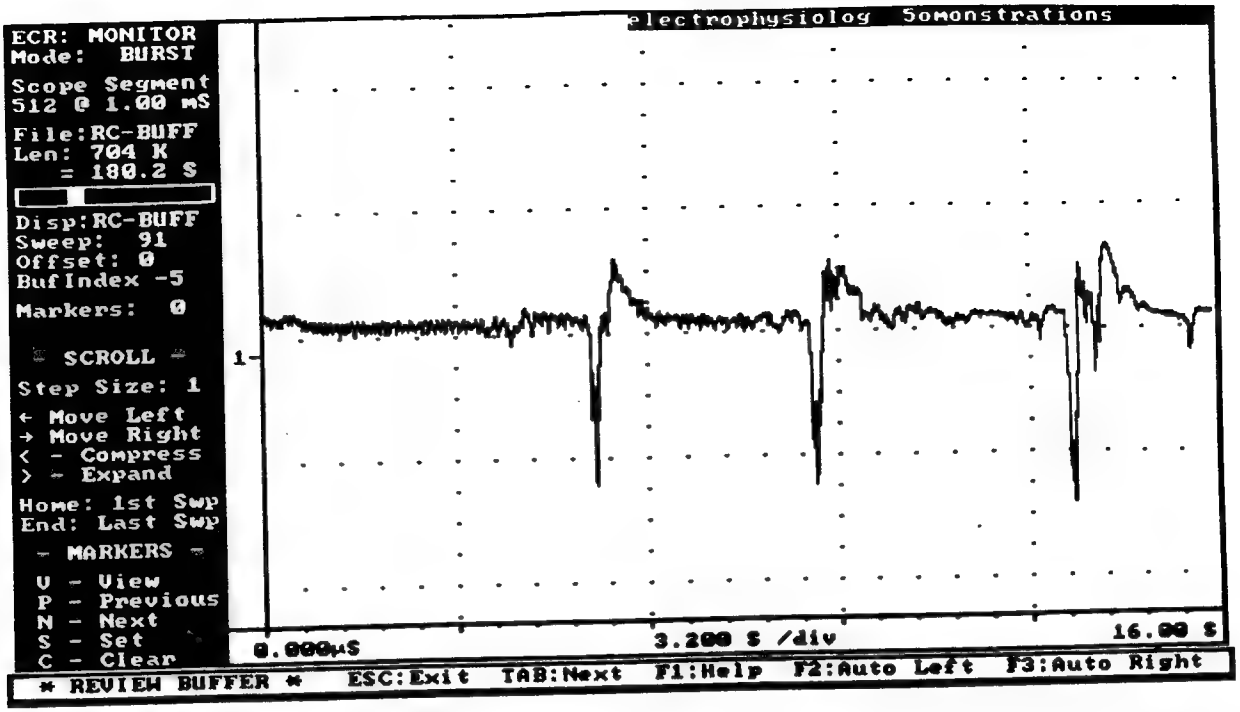

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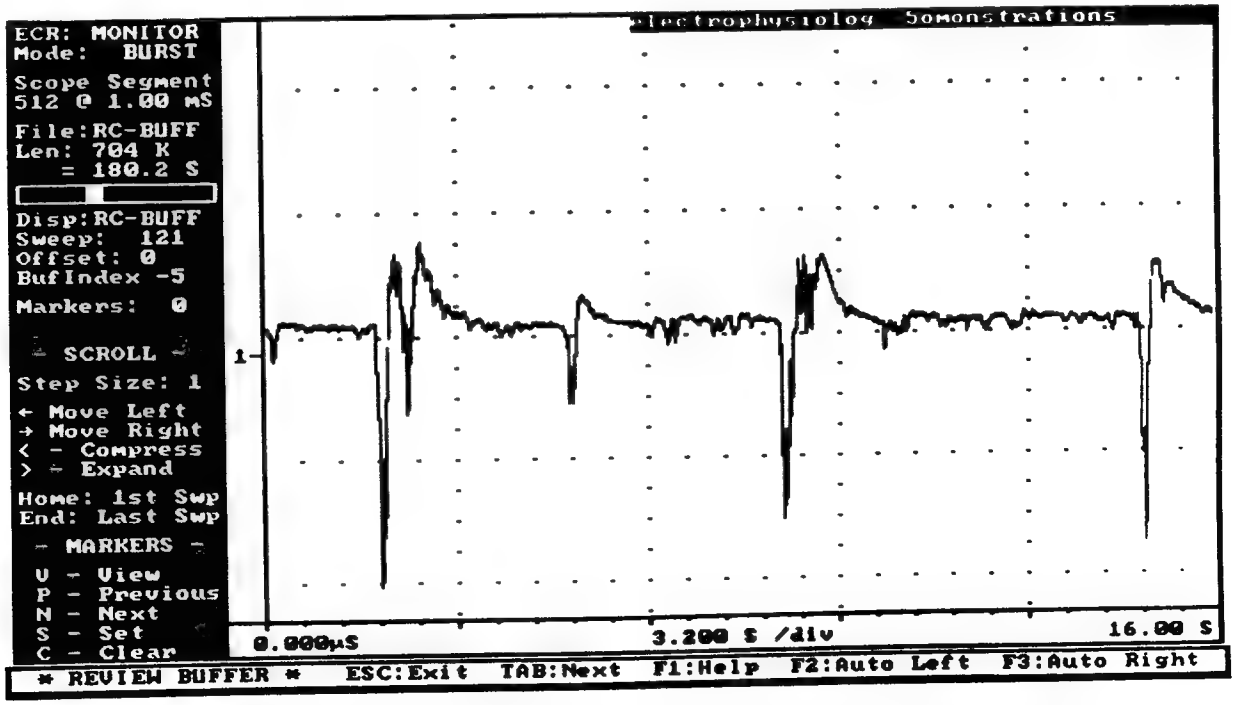

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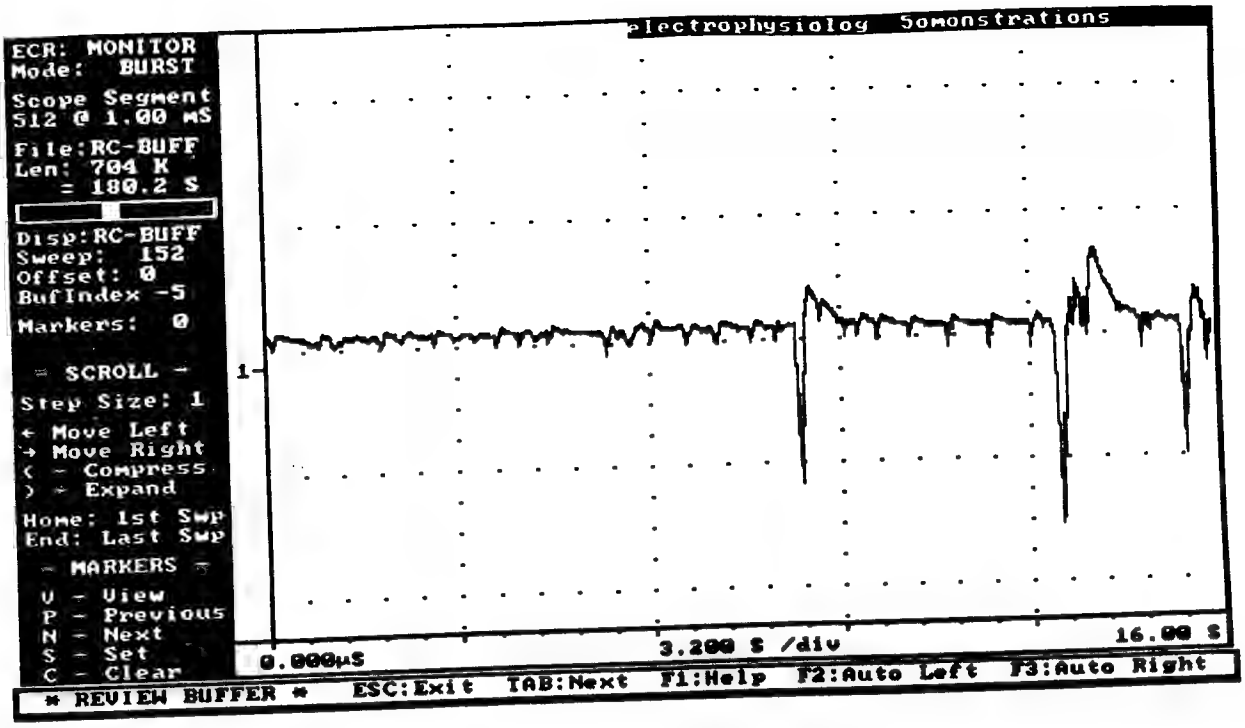

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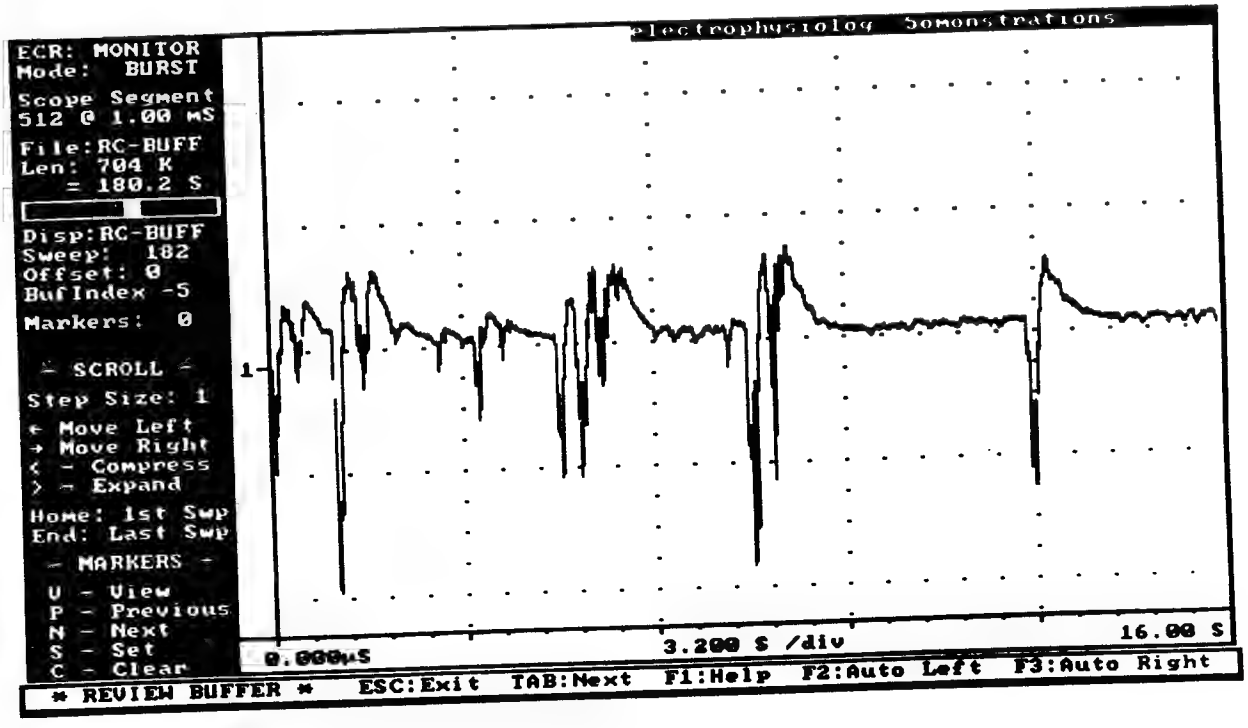

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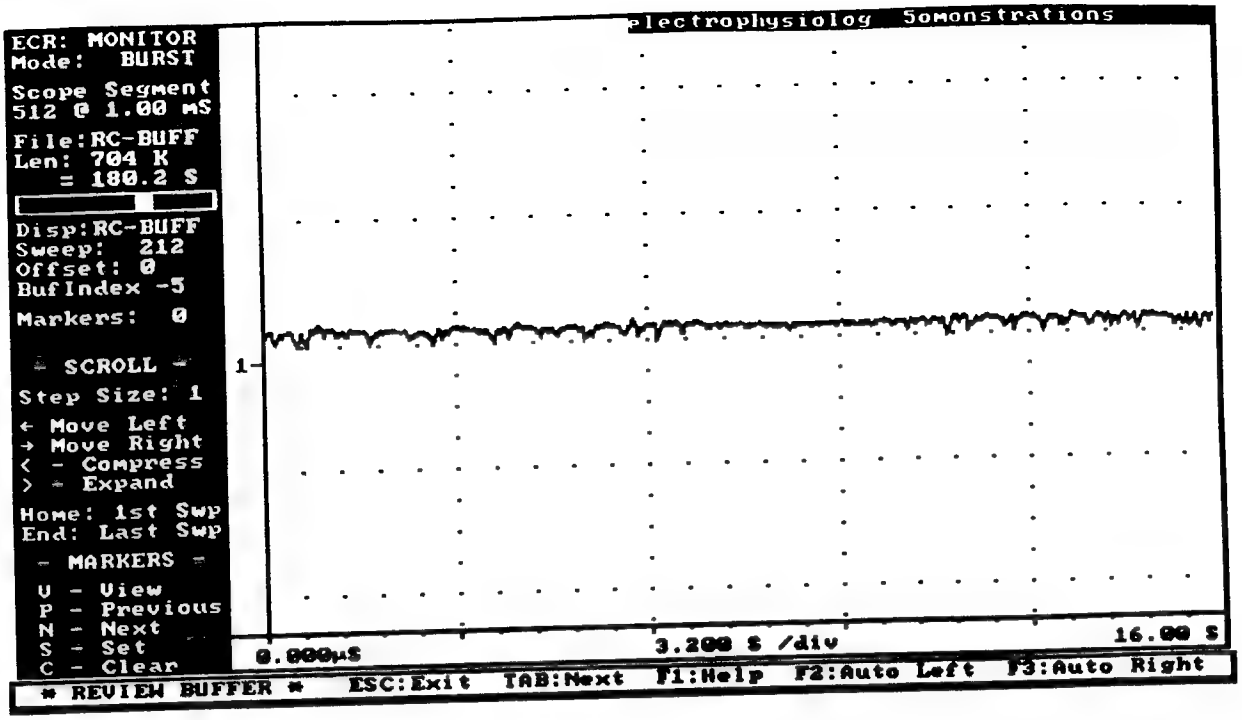

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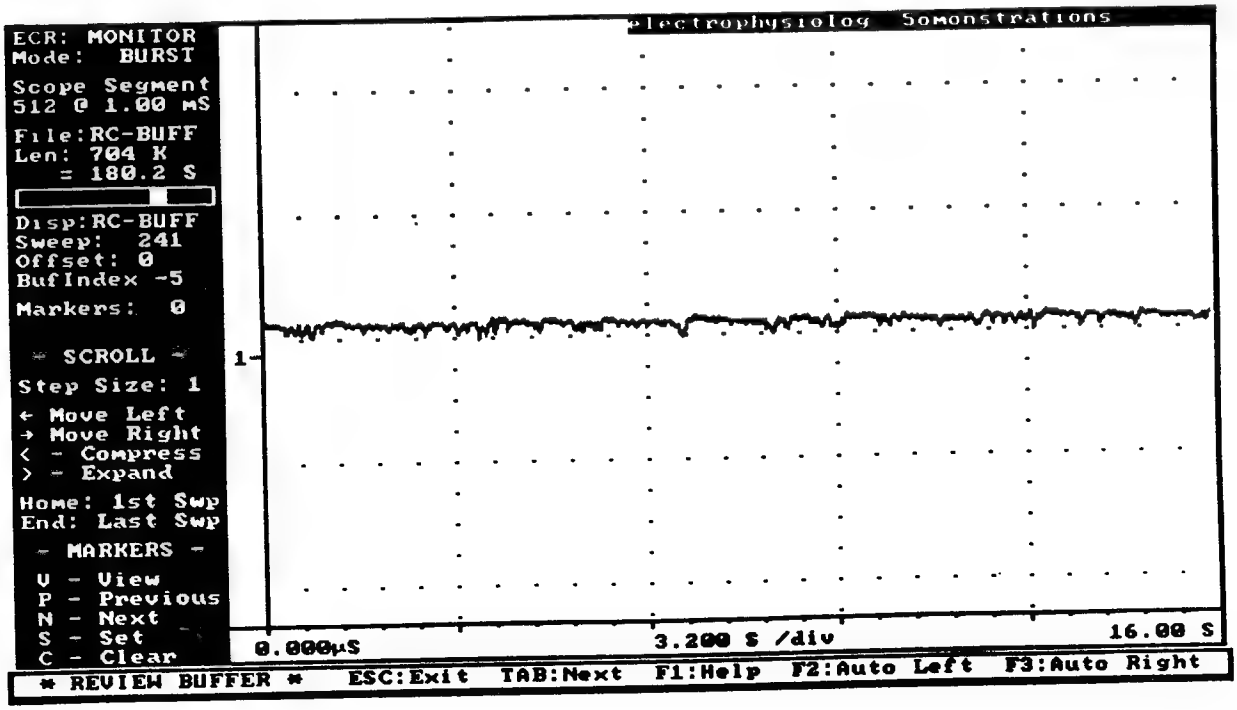
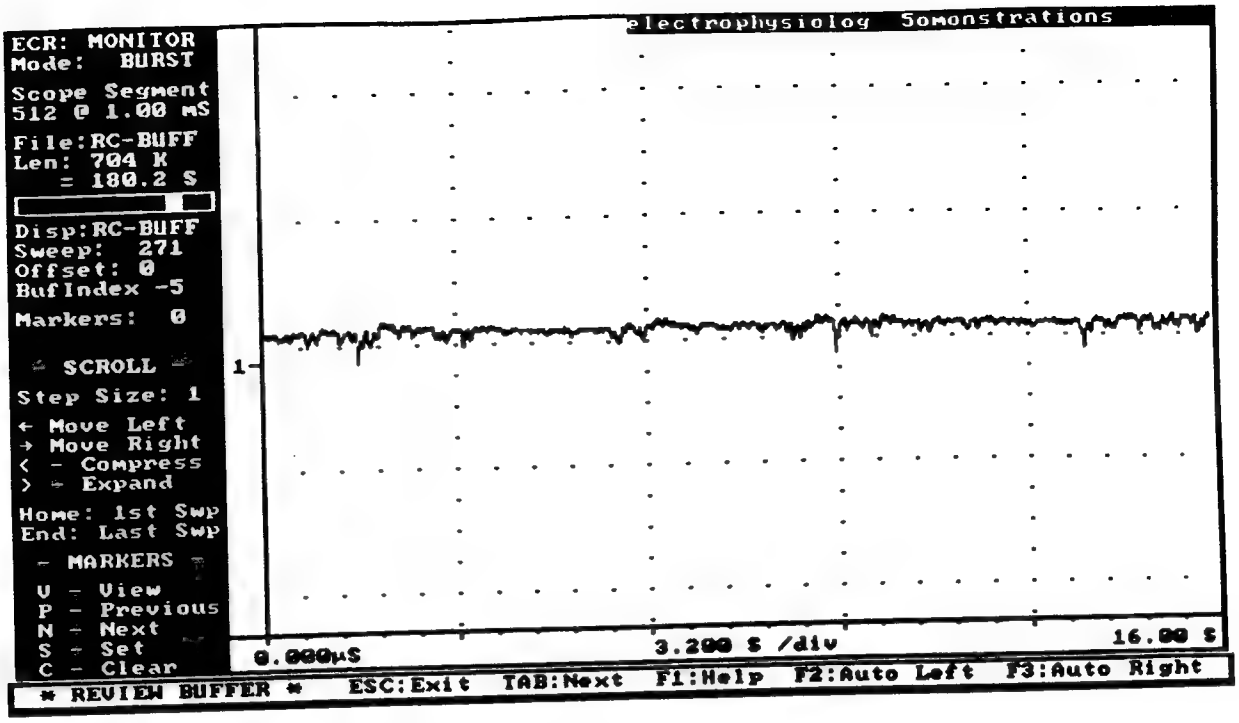


Figure 3I



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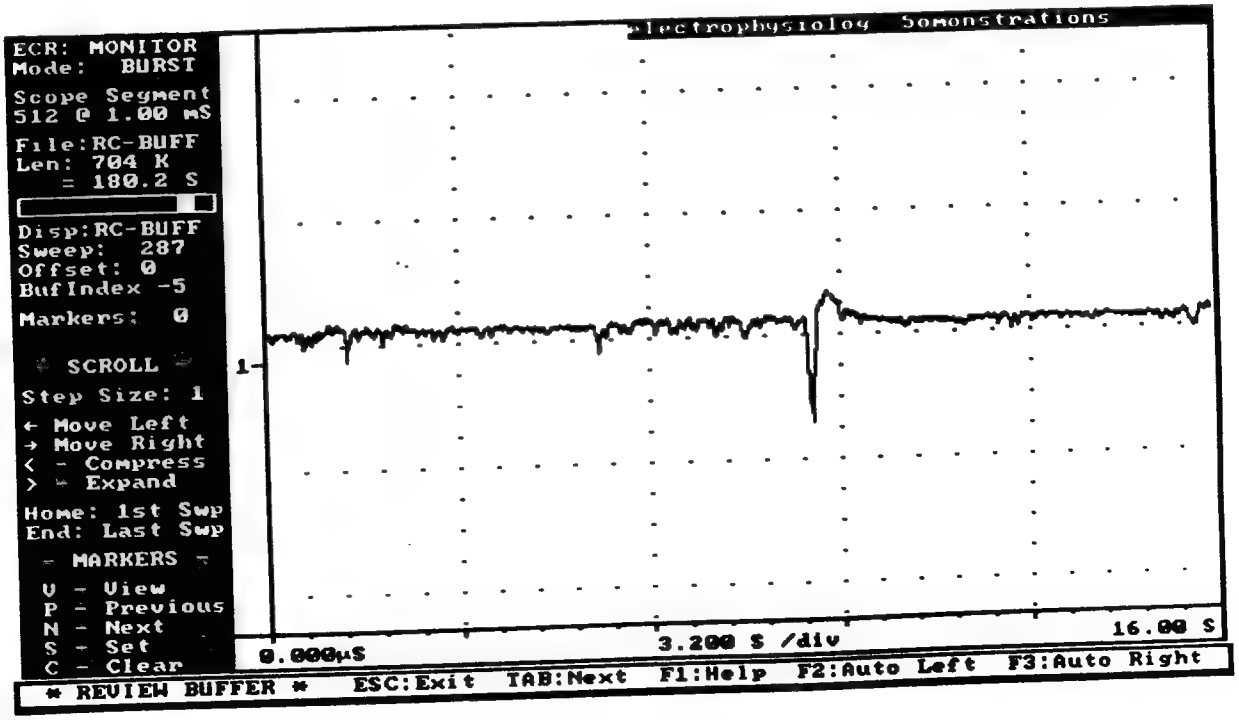

NEUROCHEMICAL PROTECTION OF THE BRAIN, NEURAL PLASTICITY AND REPAIR

PAF is a Presynaptic Mediator of Excitatory Neurotransmittor Release

FOREWARD

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Gary D Ward

Investigator Signature

ANIMAL USE
20 SEPTEMBER, 1993, THROUGH JULY, 1994

DAMD17-93-V-3013

The experimental animals used during this period for the project, Neural Responses to Injury: Prevention, Protection, and Repair, **Subproject: Neurochemical Protection of the Brain, Neural Plasticity, and Repair**, are as follows:

Species	Number Allowed	Number Used	LSU IACUC #
Rat		160	506

Investigator Signature

RESEARCH GRANT

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Appendix *(Five collated sets, No page numbering necessary for Appendix)*
Number of publications and manuscripts accepted or submitted for publication *(Not to exceed 10)*
Other items (list):

☒ Check If Appendix is Included

INTRODUCTION

Platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF), is a potent modulator of excitatory synaptic transmission and participates in synaptic plasticity since it is a messenger in rat hippocampal long term potentiation (LTP)(Clark, Happel, Zorumski, & Bazan, 1992)(Kato, Clark, Bazan, & Zorumski, 1994)(Arai & Lynch, 1992)(Cerro, Arai, & Lynch, 1990) and since it participates in human neuronal migrational defect(Hattori, Adachi, Tsujimoto, Arai, & Inoue, 1994). This modulation results in an enhanced release of the neurotransmitter, glutamate. Since glutamate is both a neurotoxin and a neurotransmitter, this enhanced release by PAF can lead to both neuronal damage and to strengthened synaptic transmission depending upon the process which initiates the production of PAF.

Previous studies in this laboratory demonstrated the enhancement of hippocampal excitatory synaptic transmission by PAF, provided evidence for a presynaptic site of action and suggested a receptor mediated process leading to synaptic enhancement(Clark, et al., 1992)(Bazan, Zorumski, & Clark, 1993). Further studies have demonstrated that PAF can cross the synapse and can lead to a long-term potentiation in the rat hippocampal slice preparation(Kato, et al., 1994).

The present work, described in this progress report, represents a further dissection of the mechanisms by which PAF mediates its effects upon rat hippocampal excitatory synaptic transmission. Using patch clamp recording techniques in rat hippocampal culture, a demonstration of an analogue of PAF crossing the synapse has been made. Software has been developed and modified in order to analyze the changes in miniature excitatory synaptic currents evoked by PAF in rat postnatal hippocampal culture. The microisland technique has been modified and established in this laboratory. Excitatory autaptic, recurrent axonal-dendritic connections, currents have been recorded. Mechanisms downstream of the PAF receptor activation have begun to be elucidated.

Screening of a human cDNA library has progressed to a tertiary screen and the resulting

clones will be sequenced. mRNA will be produced and the human brain PAF receptor will be expressed in Xenopus oocytes. If the human brain PAF receptor resembles that from rat leukocytes, then the human brain receptor will link to the native chloride channel in the Xenopus oocyte. This will give the investigator an important tool in order to study the pharmacology of the human brain PAF receptor. The potency of PAF receptor antagonists and the expected concentrations of PAF for brain receptor activation will be determined.

Experimental Methods

Rat hippocampal cultures were produced using modifications of previously established techniques(Clark, et al., 1992). Briefly, Matrigel (Collaborative Research) was used at a dilution of 1:15 and a serum free medium containing insulin, human transferrin and selenium instead of collagen and a serum containing media. Neuronal cultures were prepared from 1 to 3 day old rat pups and grown for 4 to 9 days prior to recording.

One to three MΩ patch electrodes were used to access individual neurons as described previously(Clark, et al., 1992). For the recording of miniature excitatory synaptic currents, the external solution contained (in mM) 140 NaCl, 15 KCl, 3$CaCl_2$, 2$MgCl_2$, 10Glucose, 10HEPES, .01TTX, .1bicuculline. In all recordings, the internal solution contained 140$CsMeSO_4$, 5NaCl, 10HEPES, 5 1,2-bis(o-aminophenoxylethane)-N,N,N'N'-tetra-acetic acid (BAPTA), .5$CaCl_2$. In some experiments, the internal solution contained methyl carbamyl PAF (Cayman Chemical) and in those experiments the maximal concentration of the carrier solvent, methanol, was .05%.

Screening of a commercially available cDNA library (Strategene Lambda Zap II) was performed using three 25 base probes derived from published sequences of the human heart PAF receptor and from a human leukocyte PAF receptor. Oligonucleotides were radioactively labelled

and 'lifts' from BB4 plates containing the library were performed using standard library screening techniques(Sambrook, Fritsch, & Maniatis, 1989).

Results

Patch clamp recording techniques were used in rat postnatal hippocampal culture to examine the effects of intracellular instillation of c-PAF (2μM), a nonhydrolizable form of the PAF molecule, upon miniature excitatory synaptic currents (mepscs). Since mepscs are the result of spontaneous release of excitatory transmitter from neighboring neurons, they are influenced by presynaptic mechanisms. C-PAF instillation resulted in a 1021 ± 72 % (mean ± S.E.) increase in the frequency of mepscs over that observed in other neurons in the same or sister cultures (N=10). This increase was noted to develop over 1 to 2 minutes of recording in some cells with as much as a 507% increase in frequency of mepscs from the first 30 second epoch to the third 30 second epoch. FB-0502 (2μM), a PAF receptor antagonist, dissolved in the external solution blocked the observed increase in frequency (N=2). In a similar manner, BN52021 at a concentration of 10μM (N=4) blocked the increase in frequency of mepscs but 2μM (N=3) had no significant effect.

Aminophosphonovalerate (APV), a N-methyl-D-aspartate (NMDA) receptor antagonist, at a concentration of 100μM, had no significant effect upon the c-PAF enhancement of mepscs (N=5). L-Nitroarginine, a nitric oxide synthetase inhibitor had no effect upon the c-PAF enhancement (N=4).

Pertussis toxin (1.5μg/ml) was placed in cultures for 6 hours prior to recording and two observations were made. First there was a paucity of mepscs over that observed in sister cultures (.55/sec ±.27 vs. 8.8/sec ± .9, N= 5 pertussis vs 10 not pertussis), and secondly there was no PAF effect on mepscs in pertussis toxin treated cultures Therefore, the c-PAF mediated enhancement of mepscs appears to be mediated through a pertussis toxin sensitive G-protein mediated process

Voltage-dependent calcium currents were recorded using the nystatin patch technique. The charge carrying specie in these experiments was barium in an external solution of (in mM) 140 tetraethylammonium acetate, 10 HEPES and 10 glucose. Neurons were voltage clamped at -80mV and voltage steps to -60, -20 and +20mV were performed before and after external perfusion with 2μM c-PAF. No observed increase in current amplitude was noted with the perfusion of c-PAF (N=27).

Molecular isolation of the human brain PAF receptor

Using standard oligonucleotide screening techniques, a number of potential clones of the human brain PAF receptor have been identified. At present, a tertiary screen of the cDNA library has been performed, but there is concern for some nonspecific labelling. A repeat tertiary screen will be performed and specific lambda zap II clones known to not contain the human brain PAF receptor cDNA will be used to ascertain whether or not nonspecific binding to the lambda zap vector has occurred. Recordings from naive Xenopus oocytes perfused with 10μM c-PAF reveal no activation as measured by a deviation from a holding current using two-electrode voltage clamp.

Conclusions

These data support a role for PAF as a transynaptic modulator of excitatory synaptic transmission. c-PAF, a nonhydrolizable analogue of PAF, enhanced the frequency of mepscs by crossing the synapse and acting upon neighboring presynaptic neurons. This effect was not mediated through the activation of NMDA receptors since APV appeared to have no effect upon the c-PAF enhancement of excitatory synaptic transmission. Nitric oxide production is unlikely to explain the c-PAF enhancement since L-nitroarginine did not block the c-PAF enhancement.

The c-PAF induced enhancement of excitatory synaptic transmission does appear to be mediate through a presynaptic receptor activation via a pertussis toxin G protein-mediated process. The processes leading to synaptic enhancement downstream from the PAF receptor activation, are to be elucidated in further studies of mepscs.

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NEUROCHEMICAL PROTECTION OF THE BRAIN, NEURAL PLASTICITY AND REPAIR

Neuroanatomical Correlation of PAF Antagonist-Affected Gene Expression

Quantitative Reverse Transcription Polymerase Chain Reaction

NEUROCHEMICAL PROTECTION OF THE BRAIN, NEURAL PLASTICITY AND REPAIR

NEUROANATOMICAL CORRELATIONOF PAF ANTAGONIST-AFFECTED GENE EXPRESSION.

As proposed in the original project, we have developed a quantitive RT-PCR to be able to study brain gene expression in regions of the brain during injury as well as in neural cells.

QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

Rat cyclooxygenase-2 (Cox-2) mRNA (accession no. L20085) and the rat NGFI-A gene with a 5′-flanking region (accession no. J04154) were obtained from Genbank. Polymerase chain reaction (PCR) primers were designed for NGFI-A and Cox-2 sequences using Oligo software (National Biosciences). Total rat RNA (0.5 μg) was subjected to reverse transcription (RT) for 1 hour at 37° C using MMLV reverse transcriptase (Gibco) and random hexamers as the reverse transcriptase primers. The cDNAs obtained from RT were amplified by PCR (GeneAmp RNA PCR Kit, Perkin Elmer). The PCR products were separated electrophoretically in an agarose gel (1.2%) with 1X TAE buffer. Specific bands of the expected size (770 bp for NGFI-A and 676 bp for Cox-2) were visualized by ethidium bromide staining. A Wizard PCR Preps Kit (Promega) was used to isolate and purify these bands.

Deletion mutants of the specific targets of NGFI-A and Cox-2 were generated in the following manner. RNA deletion mutants would be used as competitors in the RT-PCR reaction. Ideally, these deletion mutants would contain the same primer sites and sequence. The mutants

of the purified NGFI-A and Cox-2 cDNAs were constructed by a PCR-based strategy. Using each cDNA sequence, two novel primers containing overlapping sequences of 15 bp at their 5′ terminals were designed using Oligo software. One novel primer, UPLOW, was downstream of the original upper primer and the other one, LOWUP, was 100 bp downstream of UPLOW and closer to the original lower primer. Using purified cDNAs described above as templates and the original and novel primers as primer sets, two complementary pieces having an 85 bp deletion were generated separately by PCR. The two distinct products of the original upper-UPLOW primer set and the LOWUP-original lower primer set were mixed and amplified in the same tube under low stringency, this time using the original upper and lower primers. The PCR products were separated electrophoretically in a 1.2% agarose gel with 1X TAE buffer. Deletion mutants of the expected size (685 bp for NGFI-A mutant and 594 bp for Cox-2 mutant) were visualized by ethidium bromide staining and these bands were isolated and purified by Wizard PCR Preps Kit. The deletion mutant constructs were cloned using a TA cloning kit for further characterization with restriction enzymes and sequencing.

cRNAs were generated for both of the deletion mutant constructs. The constructs for NGFI-A and Cox-2 were amplified by using the upper primers containing a T7 polymerase promoter and the lower primers containing a 15 bp poly-T tail. The PCR products were purified using a WizardPCR Preps kit (Promega). The products of this reaction were transcribed using a T7 polymerase in vitro transcription kit. The products of this in vitro transcription reaction were treated with DNase I to remove residual DNA, and extracted with phenol/chloroform to eliminate contaminating proteins and to purify cRNAs. The cRNAs were used in the RT-PCR reaction as competitors.

Hybridization oligonucleotide probes specific for both the original and construct

sequences were designed by Oligo software. The probes were synthesized by an automatic DNA synthesizer and labeled with digoxigenin according to manufacturer's instructions (Boehringer-Mannheim). Digoxigenin-labeled probes were purified using a SurePure TLC purification Kit (USB). The yields of digoxigenin-labeled probes were estimated according to manufacturer's instructions.

The same reaction tube was used to reverse transcribe both the sample and mutant RNA together, and contained 5 mM $MgCl_2$, 1X PCR Buffer II (GenAmp Kit, Perkin Elmer), 1 mM dNTPs, 1 U/ml RNase inhibitor, 2.5 U MuLV reverse transcriptase, 0.75 μM lower primer for NGFI-A or Cox-2, 0.5 μg total RNA from rat brain, and varying amounts of RNA deletion mutant constructs in a final volume of 20 μL. The samples were incubated for 1 hour at 37° C. The products of RT reaction were denatured for 5 minutes at 99° C.

Reverse transcribed samples were amplified by PCR. The reaction tubes contained 2 mM $MgCl_2$ solution, 2.5 U AmpliTaq DNA polymerase, and 0.15 μM upper 5′-biotinylated primer in 1X PCR Buffer II at a final volume of 100 μL. The PCR reaction profile consisted of an initial incubation of 95°C for 3 minutes, 35 cycles of melting at 95° C for 1 minute, annealing at 55° C for 1 minute and extension at 72° C for 1 min, and a final incubation of 72° C for 7 minutes.

ELISA

100 ml/well of 1 mg/ml ExtrAvidin in PBSN (PBS containing 0.05% sodium azide) was dispensed into each well of 96-well microtitre plates (Nunc). These were then incubated overnight at room temperature. Residual binding capacity of coated plates were blocked for 1 h at RT by a blocking buffer, TBS-T (100 mM Tris-Cl, 150 mM NaCl, 0.05% Tween 20). Plates were washed three times with TBS-T.

After the RT-PCR reaction, PCR products were captured into the ExtrAvidin-coated wells. The PCR product (5 ml) was resuspended in 95 ml TBS-T and added to each well. Plates were incubated for 2 hours at room temperature and, at the end of incubation, washed three times with TBS-T.

Non-captured sense strand was removed by adding 100 ml 0.1 N NaOH to each well. Plates were incubated 10 minutes at room temperature and then washed once, with 0.1 X SSC and twice with 1X SSC.

Digoxigenin-labeled oligonucleotide probes (0.2 pmol/well) were dissolved in 100 ml of 1X HS (1X SSC, 0.1% N-lauroylsarcosine) for hybrindization and incubated at 42° C for 2 h. After hybridization, plates were washed three times with 1X HS and once with blocking buffer (2% (w/v) Genius Blocking reagent for nucleic acid hybridization dissolved in TBS).

The anti-DIG alkaline phosphatase was diluted in blocking buffer to a final concentration of 150 mU/ml and 100 ml of this working antibody solution was added to each well. The plates were incubated 1 hour at room temp and then washed, once with blocking buffer and twice with TBS-T.

The color substrate solution, NBT (45 ml), was prepared fresh and mixed with 35 ml X-phosphate solution and 10 ml of TSM buffer (100 mM Tris-Cl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5 at 20° C). Plates were washed once with TSM buffer and 100 ml of the NBT/X-phosphate solution in TSM buffer was added to each well. Optical densities were measured at 405 nm with a microplate reader.

NEUROCHEMICAL PROTECTION OF THE BRAIN, NEURAL PLASTICITY AND REPAIR

Traumatic Brain Injury

TRAUMATIC BRAIN INJURY

I. INTRODUCTION

An animal model for traumatic brain injury has been designed to recreate reproducible pathological conditions which allows the study of mechanisms of trauma and permit could reparative strategies or drugs with neuroprotective capabilities. Previous models for traumatic brain injury include devices which accelerate or rotate the skull (Gennerelli et. al., 1983), the controlled impact of a certain weight on the cranium (Feeney et al., 1981) or fluid percussion (McIntosh, 1989). Use of these experimental approaches results in a transient increase of intracranial pressure, reduction of pressure autoregulation leading to a late increase of intracranial pressure, and edema which might have vasogenic origin (Bruce, 1973). A transient apnea was detected immediately following these injuries which leads to a significant decrease in O_2 pressure and hypoxia (McIntosh, 1989).

Cerebral hypoxia as well as global brain ischemia triggers the release of neuroexcitatory amino acids, specifically glutamate which has been linked to further neuronal damage (Meldrum, 1985; Choi, 1990). Free fatty acids and diacylglycerol accumulation occurs at the onset of ischemia (Bazan, 1970), indicating an activation of phospholipases A_2 and C. This is one of the earliest biochemical events which may lead to neuronal damage. The activation of a specific phospholipase A_2 has been linked to arachidonic acid release and the synthesis of 1-0-alkyl-glycero-phosphatidyl choline, the precursor of platelet-activating factor (PAF) (Benveniste et al., 1982). PAF has been proposed as a second messenger and has been involved in the pathogenesis

of tissues injury (Panetta et al., 1987; Braquet et al., 1989). However, BN-52021, a PAF antagonist, has a neuroprotective effect in an ischemia-reperfusion model in the gerbil (Panetta, et al., 1987). Also, ischemia-induced release of PAF has been reported in neuronal cells (Bussolino et al., 1986). Distinct specific PAF binding sites have been demonstrated on rat brain synaptosomal membranes and in microsomal membranes (Marcheselli et al., 1990a) and specific antagonists have been identified which distinguish between the different sites (Marcheselli et al., 1994).

II. METHODS AND EXPERIMENTAL ANIMAL MODELS

A. TRAUMATIC BRAIN INJURY

1. Animals

Albino Sprague Dawley rats weighing 150 to 220 g, were anesthetized with an intramuscular injection of ketamine:xylazine (40:4 mg/kg). The scalp was exposed through to a midline incision and a hole drilled through the skull centered to the left parietal cortex, 5 mm from bregma and 4 mm from sagittal suture, without touching the dura. A 2.0 mm female Luer-Lock fitting was cemented to the skull over the hole and the animals were left to recover for 24 hours.

2. Hydraulic percussion apparatus for the traumatic brain injury model:

A percussion device was designed to produce hydraulic impacts of selected pressures in a range of 0.1 to 2.0 atm. The percussion system (see figure 1) consists of a plexiglass cylinder 11 cm long and 6.0 cm OD. The metal pendulum is dropped from a predetermined height,

according to the amount of pressure desired, striking a piston inside the cylinder. The cylinder is connected to the Leuer-lock connector, previously cemented to the rat skull, with a 2 mm ID thick-walled plastic tubing containing artificial CSF (cerebral spinal fluid) equilibrated at 37° C. The cylinder contains colored hydraulic fluid. The pendulum has a locking device which permits the pendulum to be blocked in the resting position so that a single impact is produced. The intensity of the injuries produced are in direct proportion to the pressure delivered by the piston and is detected by a pressure transducer using data acquisition software.

2. Tissue preparation

Animals were killed by decapitation after light ether anesthesia at the time of injury or after different recovery periods. The brain was rapidly dissected, and brain cortex, hippocampus, and brain stem were removed on an ice cold dissection board. Tissues were homogenized with a Polytron type homogenizer, in a buffer containing 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% n-lauroyl sarcosine, and 0.1 mercaptoethanol, pH 7.0. Samples were kept -40° C until RNA extraction.

B. VASOGENIC BRAIN INJURY

A vasogenic brain injury model has been tested as a model complementary to traumatic brain injury. Our hypothesis states that, through induction of gene expression and release of glutamate, PAF sets changes in motion which lead to brain damage. Therefore, pretreatment with novel PAF antagonist should have neuroprotective effect.

Vasogenic brain injury was generated by the placement of a liquid nitrogen-cooled probe

on the rat skull for one min. The probe, a brass rod, 9 mm in diameter and 25 mm in length, with a convened tip (to fit the curved surface of the skull), is attached to a larger brass rod (acting as a heat sink), 16 mm in diameter and 53 mm in length. This is bound to a 30 cm stainless steel handle, insulated toward its end to avoid injuries to the technician. The probe was immersed in liquid nitrogen until applied to the skull.

1. Animals

Albino Sprague Dawley rats weighing 150 to 220 g were anesthetised with ether. An incision to expose the skull was made along the midline of the scalp and the probe was rapidly applied onto the right fronto-parietal region for one minute. The animals were left to recover and killed at different times after injury (0, 1, 3, 6, 12, and 24 hours). Sham-operated animals were controls on which the surgery had been performed but not the probe application.

Brain tissues were rapidly dissected on an ice-cold dissection board, and brain cortex and hippocampus were removed rapidly. Tissues are homogenized with a Polytron type homogenizer in 4 ml buffer containing 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5 % n-lauroyl sarcosine, and 0.1 mercaptoethanol, pH 7.0. Samples were kept -40° C until RNA extraction.

2. Pretreatment of laboratory animals with PAF antagonists

Because some PAF antagonists diffuse through the blood-brain barrier inadequately, intracerebroventricular injections (icv) were performed. The drugs were dissolved in DMSO, and 2 µl per site (30 µg/animal) were injected 15 min before injury. A calibrated canulae attached to the needle of a Hammilton syringe permited injections to reach an exact depth of 4.5

mm. The rats were anesthetized with ether and the skull exposed by an incision on the scalp along the midline. Two burrows on either side of the midline were produced with a dental drill (1.5 mm diameter bit). The burrows were 5 mm from the midline and 5 mm from occipital-parietal suture.

3. RNA extraction and Northern blot analysis

Total RNA from brain regions was isolated using the guanidinium-thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (1987). After precipitation, the purified RNA extracts were resuspended in DEPC (diethyl-pirocarbonate) treated water and RNA quantified by spectrophotometry. Gel electrophoresis of RNA (5 µg per lane) is performed under denaturing conditions on a 1.2% agarose gel. RNA was transferred to Hybond-N^+ nylon membranes (Amersham, Arlington Heights Illinois) followed by hybridization at 42° C with [^{32}P]-labeled DNA probes for Zif-268, Tis-10, c-fos, jun-b, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The [^{32}P]-DNA probes were obtained by random primer extension from cDNA inserts of c-fos (Curran et al., 1987), zif-268 (Millbrandt, 1987), Tis-10 (Kujubu and Herschman, 1991) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, ATTC # 57090), (Tso et al., 1985). Autoradiography or phosphor-image quantification were performed on a Biorad® instrument.

4. A new rapid detection assay for brain edema

The extravasation of [^{181}I]-albumin is one of the most sensitive methods for detecting edema generation. However, our laboroatories have modified the Evans Blue extravasation

technique (Osamu et al., 1988) by adding a spectrofluorometer attached to a HPLC system for the detection and quantification of the samples. This modification permited us to reach a sensitivity as low as 1 ng.

One hour before injury, the animal received a *iv* injection of 2 ml 2% Evans Blue in saline solution which had been filtered through a 22 µm filter immediately prior to injection. Animals were killed and brain tissues recovered at 0, 1, and 12 hours after injury. Left brain cortex was separated from right brain cortex and a 200 µl blood sample was collected. Samples were homogenized in 2 ml of 50% trichloroacetic acid. The homogenate was centrifuged at 10,000 rpm for 20 min at 4°C in JA-7.5 rotor (Beckman®). One ml samples were carefully retrieved, to avoid resuspending the loose pellet formed, and mixed with 3 ml ethanol. These samples were stored at -20° C until Evans Blue quantitative analysis.

A HPLC pump was connected to an injection port and directly to a fluorescence detector (Beckman®). Excitation wave length was 620 nm (band width 10 nm) and emission wave length was 680 nm (band width 10 nm). The pump was set at 2 ml/min, delivering 50% trichloroacetic acid:methanol (25:75). Samples (10µl) were injected every 3 min and data was captured with System Gold software, version 6.0 (Beckman®). Evans Blue standards were dissolved in 50 % trichloroacetic acid:ethanol (25:75) and a standard curve was generated for a range of 1 to 50 ng. Data are expressed as:

$$\frac{\text{µg brain Evans Blue / mg protein in the pellet}}{\text{µg plasma Evans Blue / ml plasma}}$$

C. [^{3}H]-GLUTAMATE RELEASE FROM SYNAPTIC TERMINALS

1. Subcellular fractionation.

Animals were killed by decapitation and tissues were dissected out rapidly on an ice cold dissection board. For synaptosome preparations, tissue were resuspended in a buffer containing 50 mM Tris-HCl, 2 mM EGTA, 5 mM $MgCl_2$, 0.1 mM PMSF, 250 mM sucrose, 7.4 pH, 4 °C and homogenized with 5 strokes of a glass-Teflon Potter-Elvehjem homogenizer. Cellular debris were removed with a slow speed centrifugation at 121 x g for 5 min. The supernatant was centrifuged at 1090 x g for 11 min to yield a pellet containing most of nuclear membranes and plasma membranes (P_1). An additional centrifugation at 17400 x g for 20 min yielded a pellet containing predominantly synaptosomes and mitochondria (P_2). Fractions P_1 and P_2 were resuspended in the homogenization buffer at a final protein concentration of 1 μg/μl.

2. [^{3}H]-Glutamic acid loading in synaptosomes: Assay system for compounds which may stimulate or inhibit presynaptic release of the neurotransmitter.

[^{3}H]-glutamic acid (2.5 μCi in 100 μl of saline buffer, specific activity 17.25 Ci/mmol) was added to 50 μl of enriched synaptosomes and the mixture was incubated for 10 min at room temperature. The saline buffer contained 145 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 2 mM EGTA, 10 mM HEPES, 10 mM D-glucose, pH = 7.5. After incubation, samples were diluted with 350 μl saline buffer and loaded onto the perfusion cell, a device designed and built in our facility. Figure 2 is a diagram of the system and flow cell. The cells or synaptosomes were loaded on GF/B Whatman glass fiber filters which were secured to the perfusion cell between two lucite blocks and the Buna O ring, which creates the cell body. The area of the glass fiber

filter is about 78.5 mm^2 and the death volume of the cell and tubing is approximately 0.5 ml. An HPLC pump was used to deliver saline buffer (as described earlier, but with the addition of 0.025% BSA) at a constant flow rate of 6 ml/min. A solution containing KCl or PAF (200 µl) in the concentrations later specified was injected. When the antagonist was present, a secondary pump was used for its delivery. The perfusate was sent to a radio-chromatographic detector (Radiomatic FLO-ONE Beta series A-500, Packard, Meriden, Connecticut) for on-flow detection, or to a fraction collector (Foxy, ISCO, Lincoln, Nebraska) for liquid scintillation analysis or endogenous amino acids analysis.

D. QUANTITATIVE ANALYSIS OF ENDOGENOUS AMINO ACIDS:

1. Studies on the release of endogenous neuroexitotory or inhibitory amino acids

Endogenous amino acids were converted into thio-substituted isoindoles by the o-phtaldialdehyde (OPA) reaction of Lindroth et al. (1979). The derivatives were immediately separated by HPLC chromatography on a Microsorb™ AAAnalysis™ type 0 column (4.6 mm ID x 10 cm length) provided with a guard column (4.6 mm ID x 1.5 cm length). Column and guard column were loaded with C18 reserved batch, particle size 3 µm (Rainin, Woburn, Massachusetts). The fluorescence detector (Beckman AI 406/fluorescence 157, Beckman, San Ramon, CA) had an excitation wavelength of 305-395 nm, an emission filter wavelength of 420-650 nm, a 9 µl flow cell, and a sensitivity range of 0.1 to 10 pM. The HPLC pumps (Beckman 126, Beckman, Fullerton, CA) delivered a gradient from a ratio of 85:15 (solvent A:solvent B) to 0:100 (solvent A:solvent B) over 45 min with a flow rate of 1 ml/min. Solvent A was 0.1 M

NaOAcetate/methanol/tetrahydrofuran (95/4.5/0.5) which had been filtered and saturated with He. Solvent B was methanol. Amino acid standards (Sigma, St. Louis, MO) were individually resuspended in 50% methanol in water to a final concentration of 1 pM/µl. Sample derivatization was as described by Jones et al. (1981). Data acquisition and analysis were performed with a System Gold™ Version 7.0 software (Beckman, Fullerton, CA).

III. RESULTS

The time course analysis of mRNA expression of Tis-8 (or Zif-268), which is a class of transcription factors which encode a Zinc finger-type protein, is shown in Figure 3. We also studied the Tis-10 (or COX-2) gene which expresses a class of inducible cyclooxygenase. This gene is distinct from COX-1 which is defined as a constitutive prostaglandin H synthase or cyclooxygenase. Both Tis-8 and Tis-10 act as early responsive genes. Our findings show that Tis-8 reaches its maximum expression between 0 and 1 hour after injury, dropping to basal levels 6 hours after injury. Values 1 hour after injury were twice those of basal values. The Tis-10 mRNA increased sharply up to 2 hours after injury to five times those of basal levels, and then dropped to a two-fold increase 6 hours after injury. This levels slowly continued to decrease but still exceeded basal levels 24 hours after injury. Figure 4 shows the effect of pretreating the animals with PAF antagonists or dexamethasone, a well-known glucocorticoid which has inhibitory activity on the expression of COX-2 mRNA but not COX-1 (Yamagata et al., 1993). The PAF antagonist BN-50730 inhibited the expression of Tis-8 mRNA induced by injury. Dexamethasone also partially inhibited Tis-8 mRNA expression, but BN-50730 restored the basal levels more efficiently. The effect of PAF antagonists and dexamethasone on Tis-10

mRNA expression is shown in figure 5. Both the PAF antagonist and glucocorticoid significatively inhibited Tis-10 mRNA, but significant differences between the inhibitory potency of both compounds could be seen. From Figures 3 and 4 we can conclude that the PAF antagonist capable of blocking the PAF binding sites localized on the intracellular membranes significantly inhibits the increase in mRNA levels of Tis-8 and Tis-10 induced by cryogenic injury. The decrease of mRNA levels could be the result of inhibition of mRNA expression or the increase of RNAase activity and this point is under investigation.

Vasogenic edema generated by the cryogenic injury has been studied through measurment of levels of Evans Blue extravasation in the rat brain cortex. To validate the fluorometric detection technique, standard curves were run as shown in Figure 6. It is important to point out the linearity between 1 and 50 ng. Figure 7 compares the levels of Evans Blue that penetrated the brain tissue at 1 hour and at 12 hours. Sham animals were not subjected to injury but received drug or vehicle injection. Controls animals were subjected to injury and received vehicle injections, but did not receive the drug. BN-50730, dexamethasone, and BN-50730/dexamethasone were drug-treated animals and received the cryogenic injury. One hour after injury, levels of Evans Blue were increased in brain tissue, and the treatment was partially protective from plasma content extravasation. But, after 12 hours, the levels in injured control animals increased while the drug-treated animals showed more protection. The levels in the BN-50730 treated animals were about 30% of the control/treated animals. However, the levels of Evans Blue in dexamethasone-treated animals were significantly lower, approaching those of the sham uninjured animals. These data indicate that dexamethasone provides better protection against the transportation of plasma contents into the brain tissues, protecting the brain from

vasogenic edema produced as result of a cryogenic injury. The partially protective action of BN-50730 indicates that platelet-activating factor is involved in the brain edema after cryogenic injury.

An *in vitro* model was used to investigate the mechanisms affecting PAF involvement in brain edema. A perfusion chamber was developed in which [^{3}H]-glutamic acid-loaded synaptosomes were exposed to PAF at different concentrations and PAF antagonists. Figure 8 shows the time course of [^{3}H]-glutamic acid released by 100 nM PAF, 40 mM KCl, and the PAF antagonist BN-52021. A release of 2.5% of the total [^{3}H]-glutamic acid was produced by 40 mM KCl and, with 100 nM PAF, about 1.3 % of total [^{3}H]-glutamic acid was released. The release of glutamate by PAF was transient (lasting 1.2 seconds) indicating a more specific mechanism. When synaptosomes are pretreated with BN-52021 (100 μM), the inhibition of [^{3}H]-glutamic acid release was almost complete when stimulated by injection of PAF. No changes occurred when 40 mM KCI was utilized to stimulate [^{3}H]-glutamic acid release.

Figure 9 shows the different PAF concentrations' induction of [^{3}H]-glutamic acid release, indicating a concentration dependency, which is inhibited by 1 μM BN-52021.

IV. SUMMARY

1. The techniques for the traumatic brain injury model have been refined and the apparatus has been improved.

2. The vasogenic brain injury model has been implemented, tested, and data obtained with this model indicates that the transcription of the gene Tis-10 (or COX-1) is activated by the injury, and pretreatment with the PAF antagonist BN-50730 is able to inhibit the increased levels of

mRNA. The early gene Zif-268 (or Tis-8) is also activated in this injury model. PAF antagonists are able to inhibit Tis-8 mRNA expression. The relative rate of increase of Tis-10, when compared with Tis-8, is higher and is sustained for up to 24 hours. When comparing the PAF antagonist BN-50730 with dexamethasone, BN-50730 was more efficient in lowering the levels of mRNA to basal levels.

3. The technique developed to study the vasogenic brain edema has yielded data supporting PAF involvement. The levels of Evans Blue in the damaged brain tissues were minimized when animals were pretreated with PAF antagonists. Dexamethasone was found to protect against this condition more efficiently than BN-50730.

4. Another approach to correlate PAF involvement in brain damage is to study the [^{3}H]-glutamic acid release from synaptosomes in a perfusion chamber. The transient PAF-induced release of [^{3}H]-glutamic acid from brain cortex synaptosomes was almost completely inhibited by the PAF antagonist BN-52021. Amino acid release by KCl was not affected by BN-52021

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Figure 1

Hydraulic Percussion Model for Traumatic Brain Injury

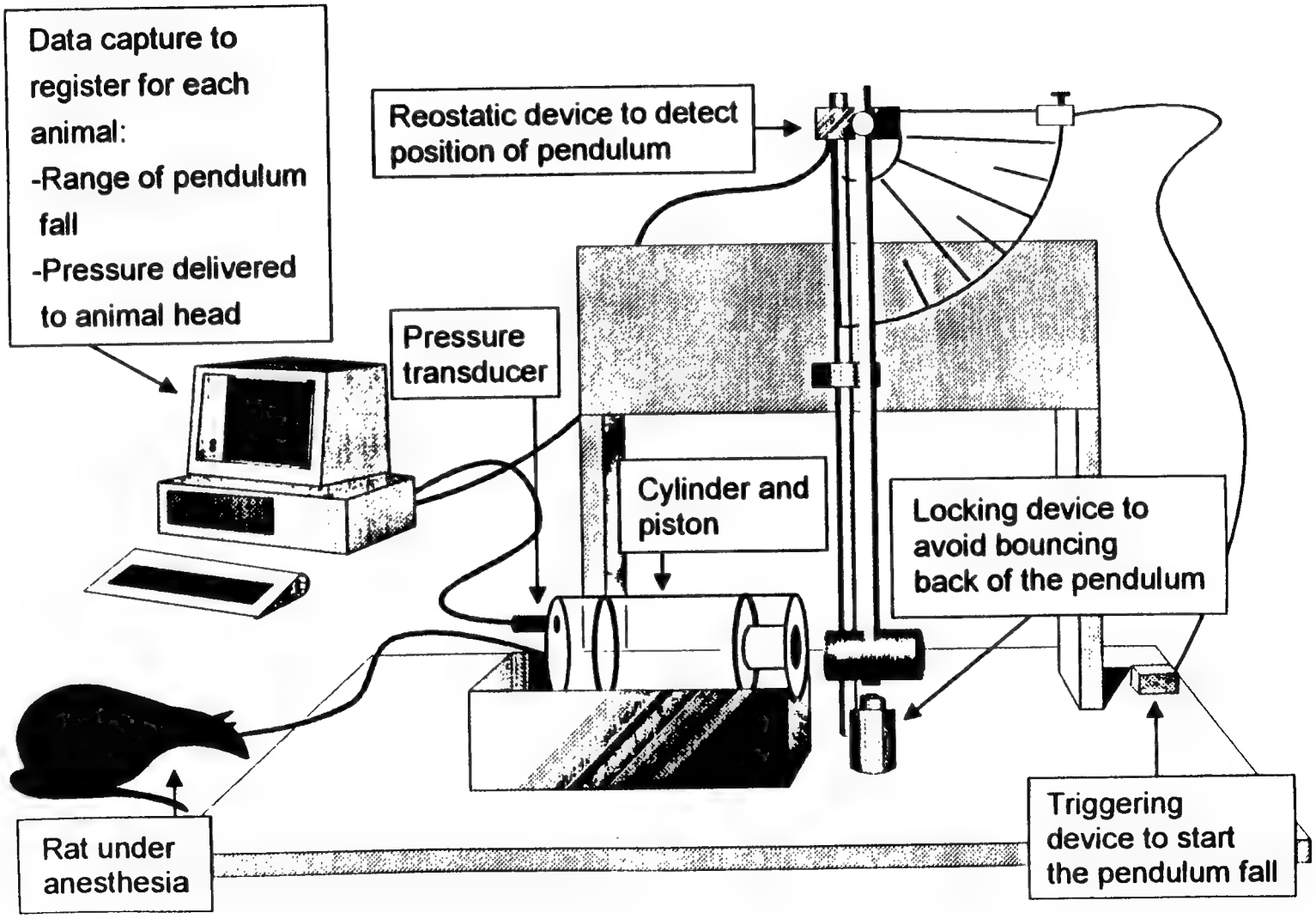
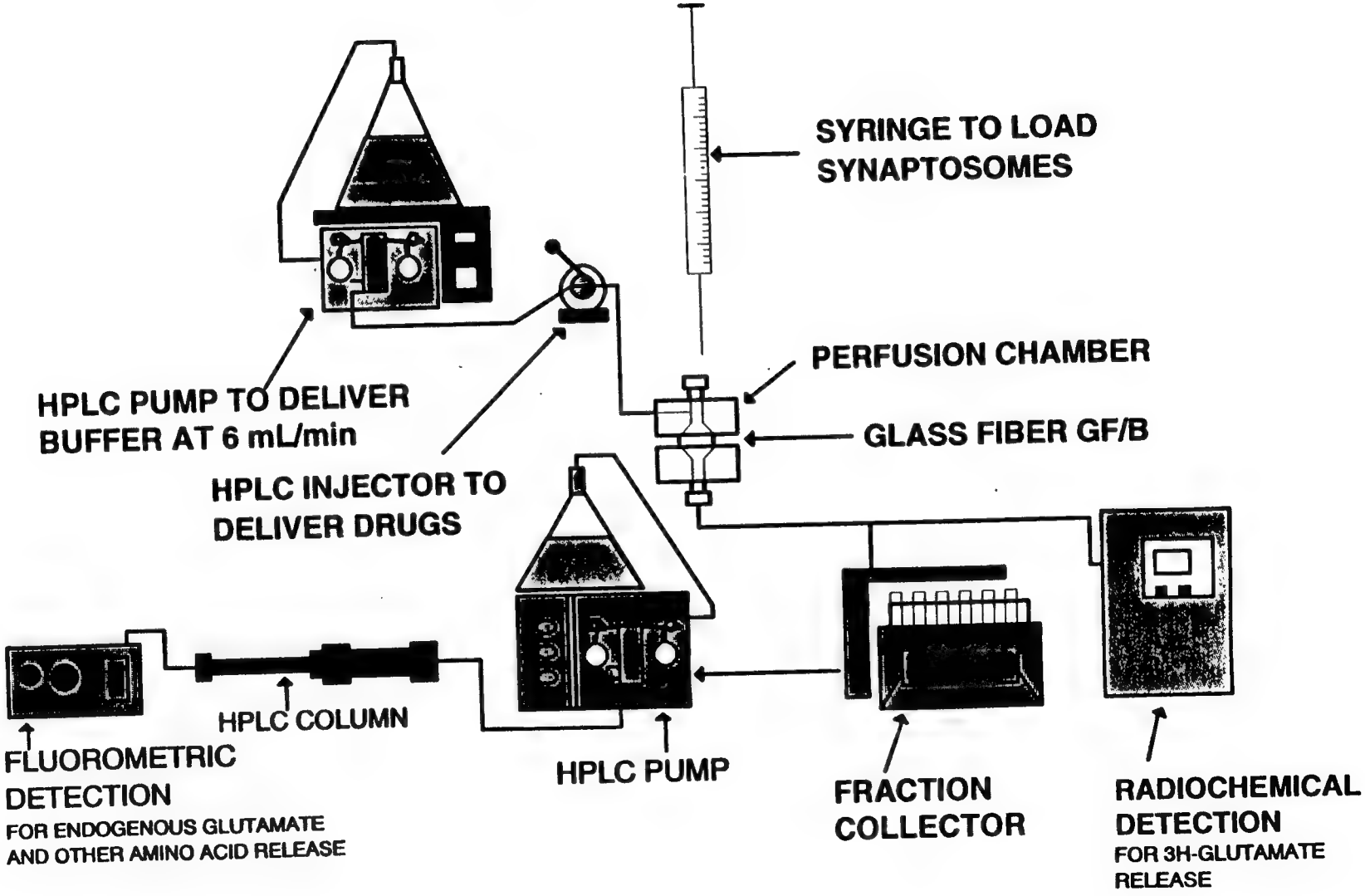


FIGURE 2

ASSAY FOR EXCITATORY AMINO ACID NEUROTRANSMITTERS RELEASE FROM SYNAPTOSOMES



TIS-10, AND TIS-8 mRNA EXPRESSION IN RAT BRAIN AFTER CRYOGENIC INJURY

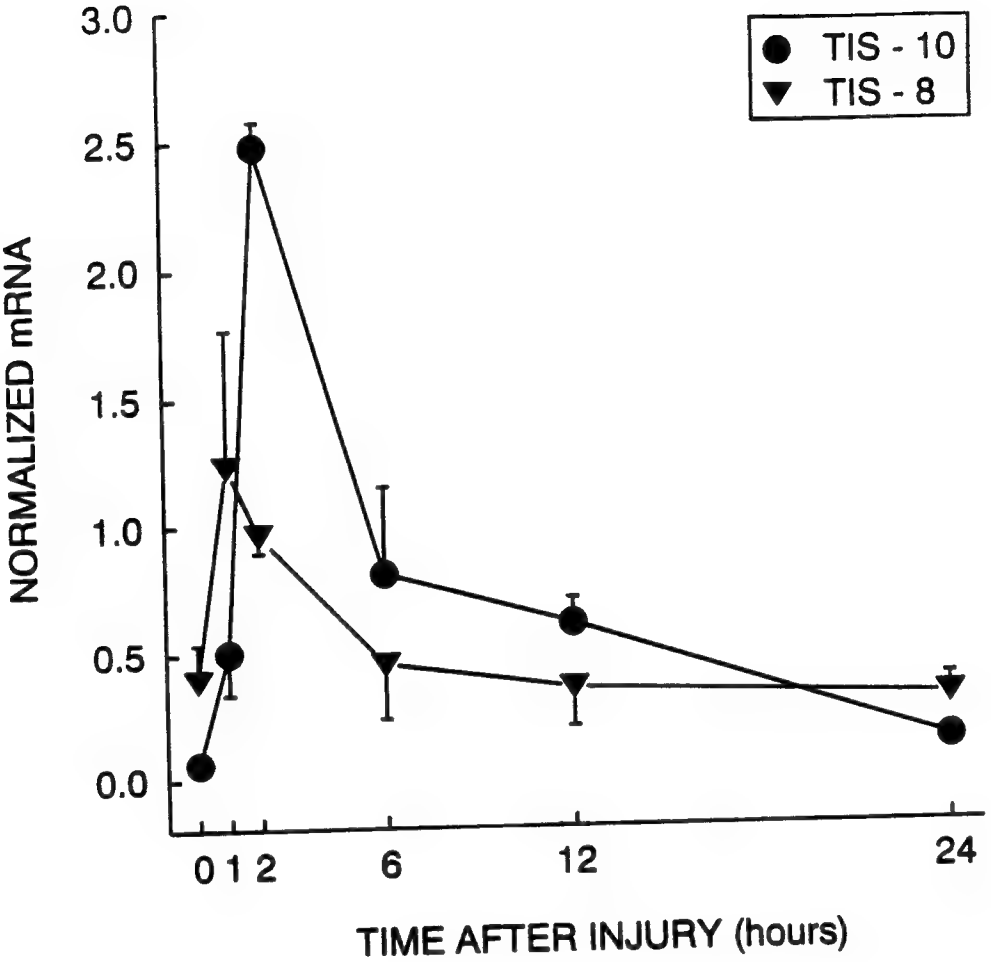


FIGURE 3 : After vasogenic injury at specified times, right rat brain cortex (injured side) were dissected, mRNA extracted and northern blot analysis performed. Blots were hybridized for Tis-8, and Tis-10 mRNA. Target genes were screened in conjunction of GAPDH as housekeeping gene. Data obtained by phosphor-image quantification, was normalized with GAPDH mRNA.

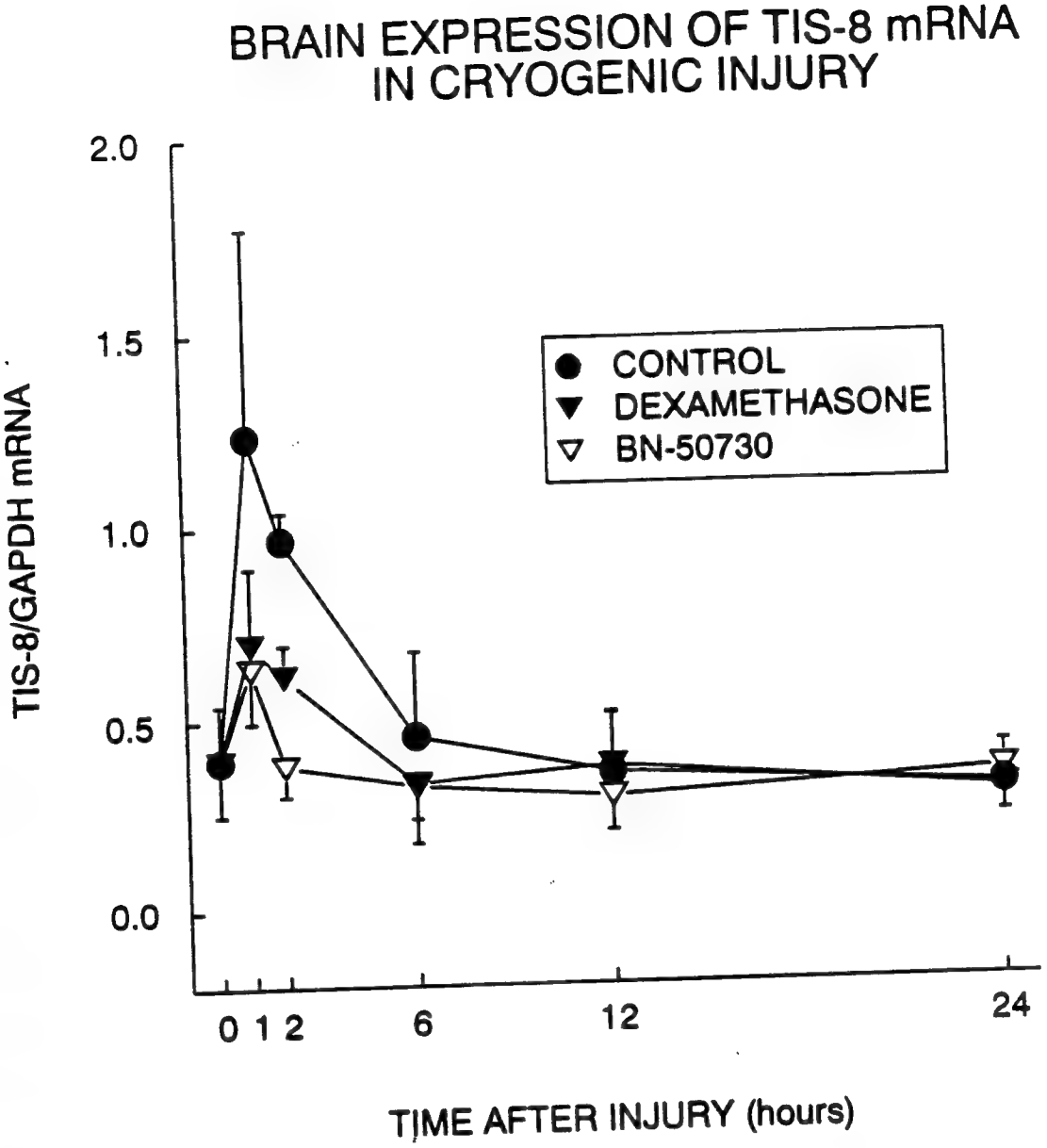


FIGURE 4: Experimental conditions were as in Figure 3. Animals treated with PAF antagonist BN-50730 were injected 30 min before injury. Delivery was done by "*icv*" injection in both ventricles, single application of 30 μg/animal disolved in DMSO. Animals treated with Dexamethasone received 3 "ip" injections one each 8 hours in a volume of 50 μl. The compound in a concentration of 6.7 μg/μl, was solubilized in DMSO:saline in a proportion of (66:34).

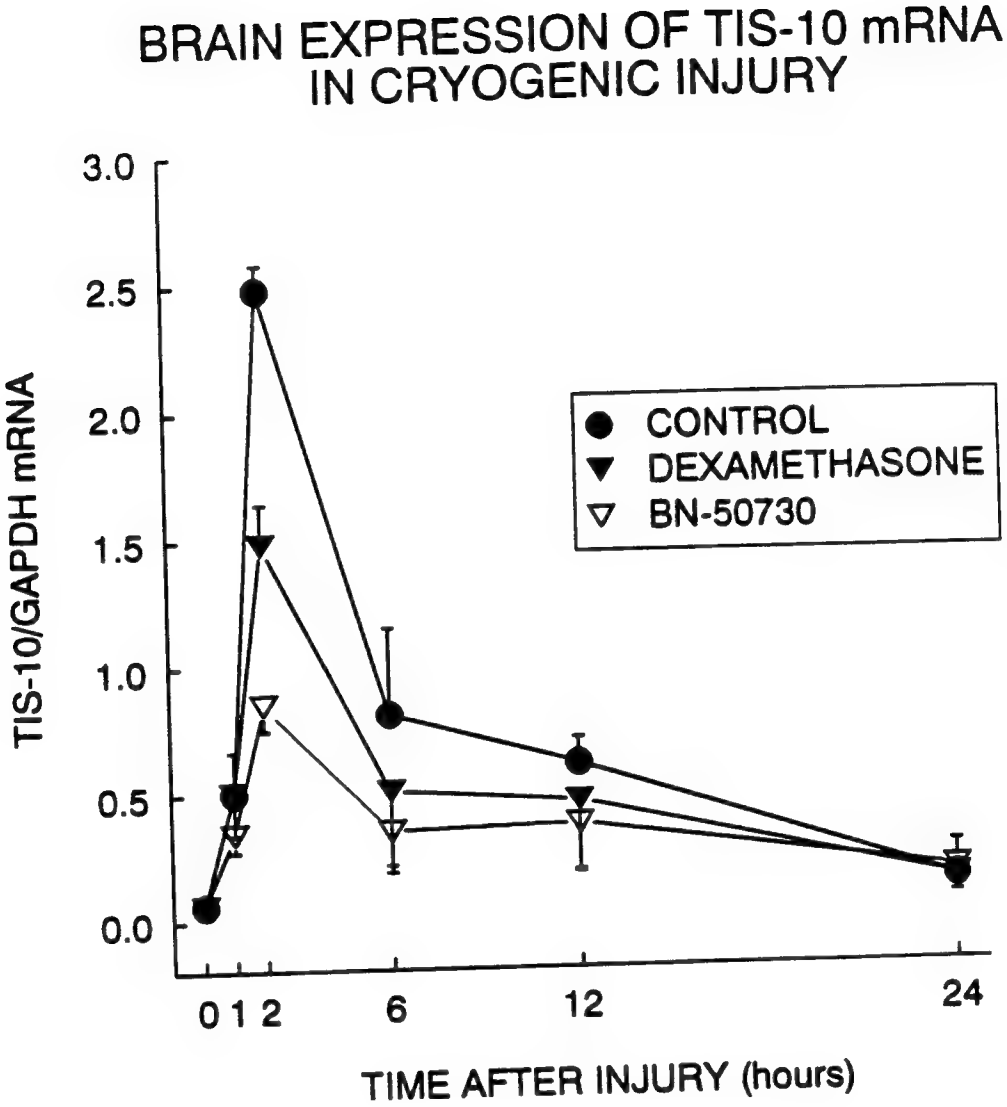


FIGURE 5 : Experimental conditions were as in Figure 3. Drug treatment as described in Figure 4.

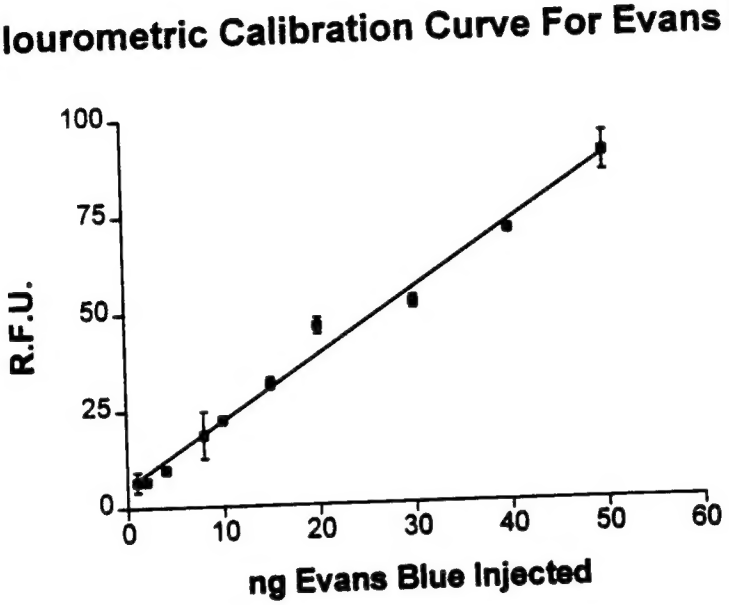


FIGURE 6 : Standard curve of Evans Blue, in a range of 1 to 50 ng, was solubilized in 50% trichloroacetic acid:ethanol (25:75). Injections of Standard in a volume of 10 μl were performed through a HPLC port running at a flow rate of 2 ml/min of a solution containing 50% trichloroacetic acid:methanol (25:75). The system was connected to a fluorescence detector. Excitation wave length was 620 nm, and emission wave length was 680 nm.

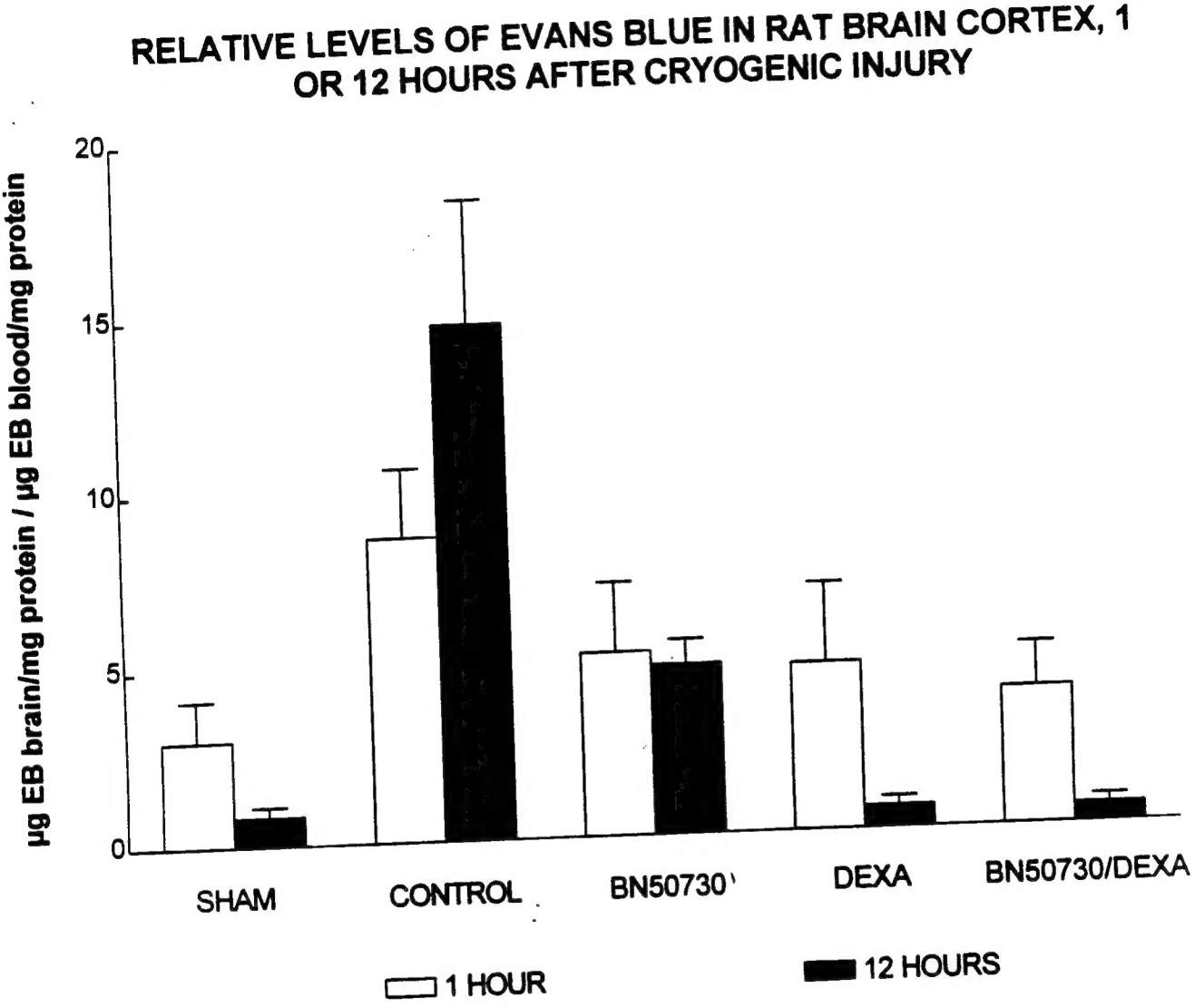


FIGURE 7 : One hour before vasogenic injury, animals were "*iv*" injected with 2 ml of 2% Evans Blue in saline solution. After 1 or 12 hours of the injury, right rat brain cortex (injured side) were dissected, and homogenized in 50% trichloroacetic acid. Extracts 10 μl volume were injected in a HPLC system for Evans Blue detection, as described in Figure 6.

TIME COURSE OF 3H - GLUTAMIC ACID RELEASE FROM RAT HIPPOCAMPUS SYNAPTOSOMES AFTER PAF INJECTION

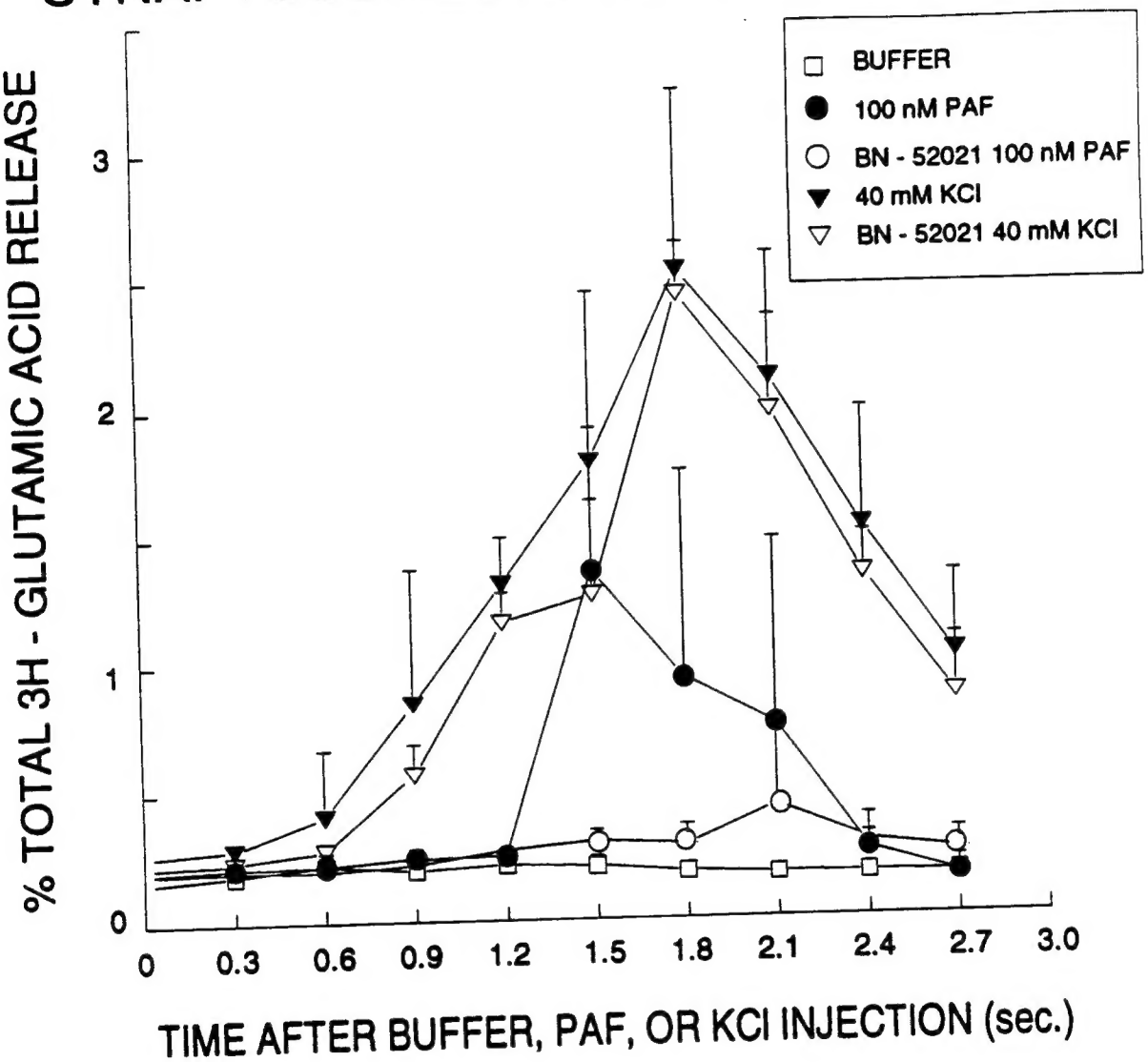


FIGURE 8 : Rat brain hippocampus synaptosomes, obtained by subcellular fractionation. 50 μg of protein which has previously loaded with ^{3}H-glutamic acid, were placed into a perfusion cell. Buffer at a flow rate of 6 ml/min at room temperature was perfused through the cell. Two minutes after cell loading, 200 μl PAF or KCl solution were injected through the injection port. When PAF antagonist treatment was performed, saline buffer containing 1 μM BN-52021 was perfused through the cell. Efluents from flow cells were directed to a radiochemical detector, for ^{3}H detection.

PAF INDUCED 3H-GLUTAMIC ACID RELEASE FROM RAT HIPPOCAMPUS SYNAPTOSOMES

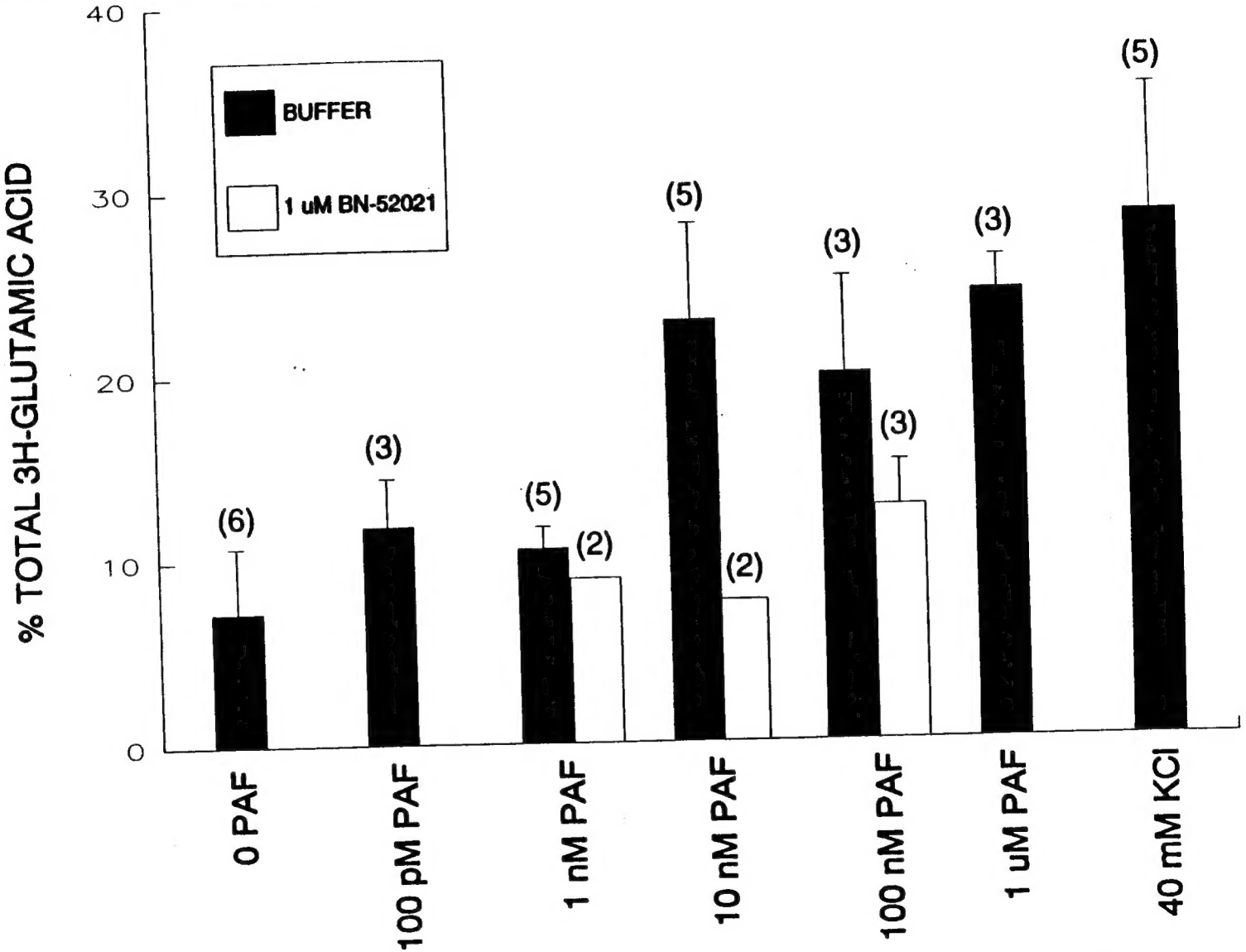


FIGURE 9 : Different PAF concentrations were used to induce ^{3}H-glutamic acid release from rat hippocampus synaptosomes. When PAF antagonist was used, 1 μM BN-52021 in saline buffer was perfused through the cell.